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(71) Applicant *(for all designated States except US)*: THE ENDOWMENT FOR RESEARCH IN HUMAN BIOLOGY, INC. [US/US]; Seeley G. Mudd Building, Room 105, 250 Longwood Avenue, Boston, MA 02115 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: HU, Guo-fu [CN/US]; Apartment 5, 551 Brookline Avenue, Brookline, MA 02146 (US). VALLEE, Bert, L. [US/US]; Apartment 712, 300 Boylston Street, Boston, MA 02116 (US).

(74) Agents: ANTLER, Adriane, M. et al.; Pennie & Edmonds LLP, 1155 Avenue of the Americas, New York, NY 10036 (US).

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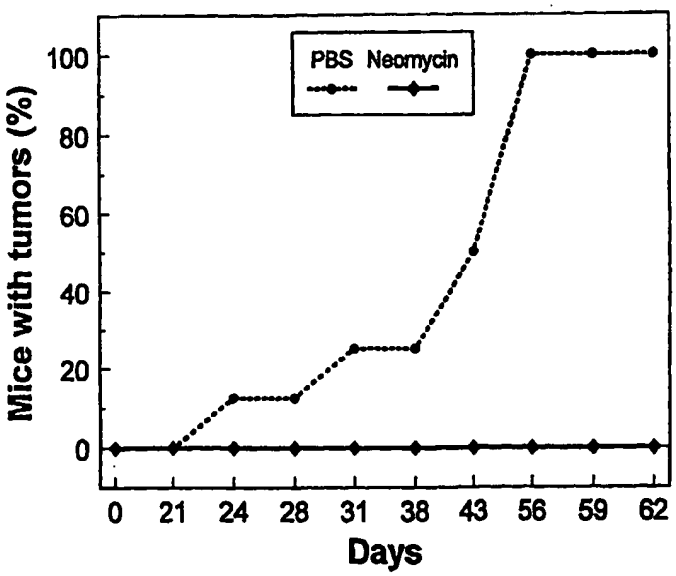
(54) Title: USE OF NEOMYCIN FOR TREATING ANGIOGENESIS-RELATED DISEASES



(57) Abstract

The present invention is directed to using neomycin or an analogue thereof as a therapeutic agent to treat angiogenesis-related diseases, which are characterized by excessive, undesired or inappropriate angiogenesis or proliferation of endothelial cells. The present invention is also directed to pharmaceutical compositions comprising: (a) neomycin or an analogue and, optionally, (b) another anti-angiogenic agent or an anti-neoplastic agent. The present invention is further directed to a method for screening neomycin analogues having anti-angiogenic activity. A preferred embodiment of the invention relates to using neomycin to treat subjects having such diseases.

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AMENDED CLAIMS
[received by the International Bureau n 29 December 1999 (29.12.99);
original claims 1, 14-21, 23, 25, 36, 39-43, 56, 64 and 75-81 amended;
remaining claims unchanged (20 pages)]

1. A method of inhibiting pathological angiogenesis or proliferation of endothelial cells in a subject, which method comprises administering to the subject an amount of neomycin or an analogue thereof sufficient to inhibit pathological angiogenesis or proliferation of endothelial cells.

2. The method according to claim 1, wherein the neomycin analogue is

(a) neomycin A, neomycin B, or neomycin C;

(b) a complex comprising neomycin A, neomycin B, or neomycin C;

(c) an aminoglycoside having a structure substantially similar to that of neomycin A, neomycin B or neomycin C;

(d) a chemical or biological breakdown product of neomycin A, neomycin B or neomycin C;

(e) a derivative of neomycin A, neomycin B or neomycin C; or

(f) a naturally-occurring precursor to neomycin A, neomycin B or neomycin C.

3. The method according to claim 2, wherein the neomycin analogue comprises a substituted-2-deoxystreptamine (2-DOS) linked to two to four sugars, wherein each sugar is a pentose or hexose.

4. The method according to claim 3, wherein the neomycin analogue is a member of the neomycin, paromomycin or lividomycin aminoglycoside family.

5. The method according to claim 4, wherein the neomycin analogue comprises a glucosyl residue attached to the 4 position of the 2-DOS moiety, which glucosyl residue comprises an amino group at each of the 2 and 6 positions.

6. The method according to claim 4, wherein the neomycin analogue comprises a 2-DOS and a 2,6-diamino-2-6-dideoxy-D-glucose attached to the 4 position of 2-DOS.

7. The method according to claim 5, wherein the neomycin analogue is nebramine, gentamine C_1, gentamine C_2, gentamine C_{1a}, ribostamycin, or xylostasin.

8. The method according to claim 1, wherein the neomycin analogue is an inhibitor of phospholipase C.

9. The method according to claim 1, wherein the neomycin analogue is an inhibitor of nuclear translocation of an angiogenic factor.

10. The method according to claim 1, wherein the neomycin analogue is an inhibitor of endothelial cell proliferation induced by an angiogenic factor.

11. The method according to claim 1, wherein the neomycin analogue is an inhibitor of angiogenesis in the chorioallantoic membrane of chick embryo induced by an angiogenic factor.

12. The method according to claim 9, 10, or 11, wherein the angiogenic factor is an acidic fibroblast growth factor, basic fibroblast growth factor, epidermal growth factor, tumor growth factor-alpha, tumor growth factor-beta, tumor necrosis factor-alpha or vascular endothelial growth factor.

13. The method according to claim 1 in which the subject is a human.

14. The method according to claim 1 in which the pathological angiogenesis or proliferation of endothelial cells is associated with a disease selected from the group consisting of fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma,

lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma, acoustic neuroma, neurofibroma, trachoma and pyogenic granulomas.

15. The method according to claim 14 wherein the disease is breast cancer.

16. The method according to claim 14 wherein the disease is prostate cancer.

17. The method according to claim 1 in which the pathological angiogenesis or proliferation of endothelial cells is associated with a disease selected from the group consisting of acute lymphocytic leukemia and acute myelocytic leukemia, chronic leukemia, polycythemia vera, lymphoma, multiple myeloma, Waldenström's macroglobulinemia, and heavy chain disease.

18. The method according to claim 1 in which the pathological angiogenesis or proliferation of endothelial cells is associated with a disease selected from the group consisting of acne rosacea, atopic keratitis, chemical burns, contact lens overwear, corneal graft rejection, diabetic retinopathy, epidemic keratoconjunctivitis, fungal ulcers, Herpes simplex infections, herpes zoster infections, Kaposi sarcoma, lipid degeneration, marginal keratolysis, Mooren ulcer, neovascular glaucoma and retrolental fibroplasia, periphigoid radial keratotomy,

phylectenulosis, polyarteritis, protozoan infections, pterygium keratitis sicca, retinopathy of prematurity, rheumatoid arthritis, sjogrens, scleritis, Steven's Johnson disease, superior limbic keratitis, syphilis, systemic lupus, Terrien's marginal degeneration, trauma, vitamin A deficiency, and Wegeners sarcoidosis.

19. The method according to claim 1 in which the pathological angiogenesis or proliferation of endothelial cells is associated with a disease selected from the group consisting of artery occlusion, Bechets disease, Bests disease, chronic retinal detachment, chronic uveitis/vitritis, carotid obstructive disease, diabetic retinopathy, Eales disease, hyperviscosity syndromes, retinitis, choroiditis, Lyme's disease, macular degeneration, optic pits, Pagets disease, pars planitis, post-laser complications, presumed ocular histoplasmosis, pseudoxanthoma elasticum, retinopathy of prematurity, sickle cell anemia, sarcoid, Stargarts disease, syphilis, systemic lupus erythematosis, toxoplasmosis, trauma, vein occlusion, rubeosis, and proliferative vitreoretinopathy.

20. The method according to claim 1 in which the pathological angiogenesis or proliferation of endothelial cells is associated with a disease selected from the group consisting of Crohn's disease and ulcerative colitis, psoriasis, rheumatoid arthritis, sarcoidosis, hemangiomas, Osler-Weber-Rendu disease, hereditary hemorrhagic telangiectasia, and acquired immune deficiency syndrome.

21. The method according to claim 14, 15, 16, or 17 which comprises additionally administering an anti-neoplastic agent to the subject.

22. The method according to claim 21, wherein the anti-neoplastic agent is selected from the group consisting of docetaxel, etoposide, trontecan, paclitaxel, teniposide, topotecan, vinblastine, vincristine, and vindesine, busulfan, improsulfan, piposulfan, aziridines, benzodepa, carboquone, meturedepa, uredepa, altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide, chlorambucil, chloraphazine, cyclophosphamide, estramustine, ifosfamide,

mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, perfosfamide, phenesterine, prednimustine, trofosfamide, uracil mustard, carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine, dacarbazine, mannomustine, mitobronitol, mitolactol, pipobroman, temozolomide, aclacinomycinsa actinomycin F_1, anthramycin, azaserine, bleomycins, cactinomycin, carubicin, carzinophilin, chromomycins, dactinomycin, daunorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, idarubicin, menogaril, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, pirarubicin, plicamycin, porfiromycin, puromycin, streptonigrin, streptozocin, tubercidin, zinostatin, zorubicin, denopterin, edatrexate, methotrexate, piritrexim, pteropterin, Tomudex®, trimetrexate, cladribine, fludarabine, 6-mercaptopurine, thiamiprine, thioguanine, ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, doxifluridine, emitefur, enocitabune, floxuridine, fluorouracil, gemcitabine, tegafur, L-asparaginase, interferon-α, interferon-β, interferon-γ, interleukin-2, lentinan, propagermanium, PSK, roquinimex, sizofican, ubenimex, carboplatin, cisplatin, miboplatin, oxaliplatin, aceglarone, amsacrine, bisantrene, defosfamide, demecolcine, diaziquone, eflornithine, elliptinium acetate, etoglucid, fenretinide, gallium nitrate, hydroxyurea, lonidamine, miltefosine, mitoguazone, mitoxantrone, mopidamol, nitracine, pentostain, phenamet, podophyllinic acid 2-ethyl-hydrazide, procabazine, razoxane, sobuzoxane, spirogermanium, tenuzonic acid, triaziquone, 2,2',2"trichlorotriethylamine, urethan, calusterone, dromostanolone, epitiostanol, mepitiostane, testolacone, aminoglutethimide, mitotane, trilostane, bicalutamide, flutamide, nilutamide, droloxifene, tamoxifen, toremifene, aminoglutethimide, anastrozole, fadrozole, formestane, letrozole, fosfestrol, hexestrol, polyestradiol phosphate, buserelin, goserelin, leuprolide, triptorelin, chlormadinone acetate, medroxyprogesterone, megestrol acetate, melengestrol, porfimer sodium, batimastar, and folinic acid.

23. A method of inhibiting pathological angiogenesis or proliferation of endothelial cells in a subject, which method comprises administering to the subject a therapeutic amount of (a) neomycin or an analogue thereof, and (b) an anti-angiogenic

agent that is not neomycin or an analogue thereof, sufficient to inhibit pathological angiogenesis or proliferation of endothelial cells.

24. The method according to claim 23, wherein the anti-angiogenic agent is selected from the group consisting of thalidomide, 2-methoxyestradiol, endostatin, angiostatin, platelet factor-4, dextran sulfate, beta-1,3-glucan sulfate, interferon-alpha, interleukin-12, 22-oxa-1 α, 25-dihydroxyvitamin D_2, monoclonal antibody 26-2F, monoclonal antibody 36U, peptide comprising the sequence NH_2-Val-Phe-Ser-Val-Arg-Val-Ser-Ile-Leu-Val-Phe-COOH, peptide comprising the sequence NH_2-Leu-Leu-Phe-Leu-Pro-Leu-Gly-Val-Ser-Leu-Leu-Asp-Ser-COOH, human placental ribonuclease inhibitor, peptide comprising the sequence NH_2-Tyr-Ser-Val-Trp-Ile-Gly-Gly-Ser-Ile-Leu-Ala-Ser-Leu-Ser-Thr-Phe-Gln-Gln-Met-Trp-Ile-Ser-Lys-COOH, peptide comprising the sequence NH_2-Ala-Gln-Leu-Ala-Gly-Glu-Cys-Arg-Glu-Asn-Val-Cys-Met-Gly-Ile-Glu-Gly-Arg-COOH, nucleotide comprising the sequence 5'-CGGACGAATGCTTTGATGTTGTGCTGGACCAGCGTTCATTCTCA-3', anthracycline, 15-deoxyspergualin, D-penicillamine, eponemycin, fumagillin, AGM-1470, herbimycin A, rapamycin, CAI, CM101, and marimastat.

25. A pharmaceutical composition comprising a therapeutically effective amount of (a) neomycin or an analogue thereof, and (b) an anti-angiogenic agent that is not neomycin or an analogue thereof, in pharmaceutically acceptable form sufficient to suppress pathological angiogenesis or proliferation of endothelial cells in a subject.

26. The pharmaceutical composition of claim 25, wherein the neomycin analogue is

(a) neomycin A, neomycin B, or neomycin C;

(b) a complex comprising neomycin A, neomycin B, or neomycin C;

(c) an aminoglycoside having a structure substantially similar to that of neomycin A, neomycin B or neomycin C;

(d) a chemical or biological breakdown product of neomycin A, neomycin B or neomycin C;

(e) a derivative of neomycin A, neomycin B or neomycin C; or

(f) a naturally-occurring precursor to neomycin A, neomycin B or neomycin C.

27. The pharmaceutical composition of claim 26, wherein the neomycin analogue comprises a substituted-2-deoxystreptamine (2-DOS) linked to two to four sugars, wherein each sugar is a pentose or hexose.

28. The pharmaceutical composition of claim 27, wherein the neomycin analogue is a member of the neomycin, paromomycin or lividomycin aminoglycoside family.

29. The pharmaceutical composition of claim 28, wherein the neomycin analogue comprises a glucosyl residue attached to the 4 position of the 2-DOS moiety, which glucosyl residue comprises an amino group at each of the 2 and 6 positions.

30. The pharmaceutical composition of claim 29, wherein the neomycin analogue comprises a 2-DOS and a 2,6-diamino-2-6-dideoxy-D-glucose attached to the 4 position of 2-DOS.

31. The pharmaceutical composition of claim 29, wherein the neomycin analogue is nebramine, gentamine C_1, gentamine C_2, gentamine C_{1a}, ribostamycin, or xylostasin.

32. The pharmaceutical composition of claim 25, wherein the neomycin analogue is an inhibitor of nuclear translocation of an angiogenic factor.

33. The pharmaceutical composition of claim 25, wherein the neomycin analogue is an inhibitor of phospholipase C.

34. The pharmaceutical composition of claim 25, wherein the neomycin analogue is an inhibitor of endothelial cell proliferation induced by an angiogenic factor.

35. The pharmaceutical composition of claim 25, wherein the neomycin analogue is an inhibitor of angiogenesis in the chorioallantoic membrane of chick embryo induced by an angiogenic factor.

36. The pharmaceutical composition of claim 32, 34 or 35, wherein the angiogenic factor is an acidic fibroblast growth factor, basic fibroblast growth factor, epidermal growth factor, tumor growth factor-alpha, tumor growth factor-beta, tumor necrosis factor-alpha, vascular endothelial growth factor, platelet-derived growth factor, platelet-derived endothelial cell growth factor, placental growth factor, hepatocyte growth factor, platelet activating factor, insulin-like growth factor, interleukin-8, or granulocyte-colony stimulating factor.

37. The pharmaceutical composition of claim 25, further comprising a pharmaceutically acceptable carrier.

38. The pharmaceutical composition of claim 25 in which the subject is a human.

39. The pharmaceutical composition of claim 25 in which the pathological angiogenesis or proliferation of endothelial cells is associated with a disease selected from the group consisting of fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary

carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma, acoustic neuroma, neurofibroma, trachoma and pyogenic granulomas.

40. The pharmaceutical composition of claim 25 in which the pathological angiogenesis or proliferation of endothelial cells is associated with a disease selected from the group consisting of acute lymphocytic leukemia and acute myelocytic leukemia, chronic leukemia, polycythemia vera, lymphoma, multiple myeloma, Waldenström's macroglobulinemia, and heavy chain disease.

41. The pharmaceutical composition of claim 25 in which the pathological angiogenesis or proliferation of endothelial cells is associated with a disease selected from the group consisting of acne rosacea, atopic keratitis, chemical burns, contact lens overwear, corneal graft rejection, diabetic retinopathy, epidemic keratoconjunctivitis, fungal ulcers, Herpes simplex infections, herpes zoster infections, Kaposi sarcoma, lipid degeneration, marginal keratolysis, Mooren ulcer, neovascular glaucoma and retrolental fibroplasia, periphigoid radial keratotomy, phylectenulosis, polyarteritis, protozoan infections, pterygium keratitis sicca, retinopathy of prematurity, rheumatoid arthritis, sjogrens, scleritis, Steven's Johnson disease, superior limbic keratitis, syphilis, systemic lupus, Terrien's marginal degeneration, trauma, vitamin A deficiency, and Wegeners sarcoidosis.

42. The pharmaceutical composition of claim 25 in which the pathological angiogenesis or proliferation of endothelial cells is associated with a disease selected from the group consisting of artery occlusion, Bechets disease, Bests disease, chronic retinal detachment, chronic uveitis/vitritis, carotid obstructive disease, diabetic retinopathy, Eales disease, hyperviscosity syndromes, retinitis, choroiditis, Lyme's

disease, macular degeneration, optic pits, Pagets disease, pars planitis, post-laser complications, presumed ocular histoplasmosis, pseudoxanthoma elasticum, retinopathy of prematurity, sickle cell anemia, sarcoid, Stargarts disease, syphilis, systemic lupus erythematosis, toxoplasmosis, trauma, vein occlusion, rubeosis, and proliferative vitreoretinopathy.

43. The pharmaceutical composition of claim 25 in which the pathological angiogenesis or proliferation of endothelial cells is associated with a disease selected from the group consisting of Crohn's disease and ulcerative colitis, psoriasis, rheumatoid arthritis, sarcoidosis, hemangiomas, Osler-Weber-Rendu disease, hereditary hemorrhagic telangiectasia, and acquired immune deficiency syndrome.

44. The pharmaceutical composition of claim 25 in which the anti-angiogenic factor is selected from the group consisting of thalidomide, 2-methoxyestradiol, endostatin, angiostatin, platelet factor-4, dextran sulfate, beta-1,3-glucan sulfate, interferon-alpha, interleukin-12, 22-oxa-1 α, 25-dihydroxyvitamin D_2, monoclonal antibody 26-2F, monoclonal antibody 36U, peptide comprising the sequence NH_2-Val-Phe-Ser-Val-Arg-Val-Ser-Ile-Leu-Val-Phe-COOH, peptide comprising the sequence NH_2-Leu-Leu-Phe-Leu-Pro-Leu-Gly-Val-Ser-Leu-Leu-Asp-Ser-COOH, human placental ribonuclease inhibitor, peptide comprising the sequence NH_2-Tyr-Ser-Val-Trp-Ile-Gly-Gly-Ser-Ile-Leu-Ala-Ser-Leu-Ser-Thr-Phe-Gln-Gln-Met-Trp-Ile-Ser-Lys-COOH, peptide comprising the sequence NH_2-Ala-Gln-Leu-Ala-Gly-Glu-Cys-Arg-Glu-Asn-Val-Cys-Met-Gly-Ile-Glu-Gly-Arg-COOH, nucleotide comprising the sequence 5'-CGGACGAATGCTTTGATGTTGTGCTGGACCAGCGTTCATTCTCA-3', anthracycline, 15-deoxyspergualin, D-penicillamine, eponemycin, fumagillin, AGM-1470, herbimycin A, rapamycin, CAI, CM101, and marimastat.

45. A pharmaceutical composition comprising a therapeutically effective amount of (a) neomycin or an analogue thereof, and (b) an anti-neoplastic agent, in

pharmaceutically acceptable form and in an amount sufficient to treat an angiogenesis-related disease which is a tumor in a subject.

46. The pharmaceutical composition of claim 45, wherein the neomycin analogue is

(a) neomycin A, neomycin B, or neomycin C;

(b) a complex comprising neomycin A, neomycin B, or neomycin C;

(c) an aminoglycoside having a structure substantially similar to that of neomycin A, neomycin B or neomycin C;

(d) a chemical or biological breakdown product of neomycin A, neomycin B or neomycin C;

(e) a derivative of neomycin A, neomycin B or neomycin C; or

(f) a naturally-occurring precursor to neomycin A, neomycin B or neomycin C.

47. The pharmaceutical composition of claim 46, wherein the neomycin analogue comprises a substituted-2-deoxystreptamine (2-DOS) linked to two to four sugars, wherein each sugar is a pentose or hexose.

48. The pharmaceutical composition of claim 47, wherein the neomycin analogue is a member of the neomycin, paromomycin or lividomycin aminoglycoside family.

49. The pharmaceutical composition of claim 48, wherein the neomycin analogue comprises a glucosyl residue attached to the 4 position of the 2-DOS moiety, which glucosyl residue comprises an amino group at each of the 2 and 6 positions.

50. The pharmaceutical composition of claim 49, wherein the neomycin analogue comprises a 2-DOS and a 2,6-diamino-2-6-dideoxy-D-glucose attached to the 4 position of 2-DOS.

51. The pharmaceutical composition of claim 49, wherein the neomycin analogue is nebramine, gentamine C_1, gentamine C_2, gentamine C_{1a}, ribostamycin, or xylostasin.

52. The pharmaceutical composition of claim 45, wherein the neomycin analogue is an inhibitor of nuclear translocation of an angiogenic factor.

53. The pharmaceutical composition of claim 45, wherein the neomycin analogue is an inhibitor of phospholipase C.

54. The pharmaceutical composition of claim 45, wherein the neomycin analogue is an inhibitor of endothelial cell proliferation induced by an angiogenic factor.

55. The pharmaceutical composition of claim 45, wherein the neomycin analogue is an inhibitor of angiogenesis in the chorioallantoic membrane of chick embryo induced by an angiogenic factor.

56. The pharmaceutical composition of claim 52, 54 or 55, wherein the angiogenic factor is an acidic fibroblast growth factor, basic fibroblast growth factor, epidermal growth factor, tumor growth factor-alpha, tumor growth factor-beta, tumor necrosis factor-alpha, vascular endothelial growth factor, platelet-derived growth factor, platelet-derived endothelial cell growth factor, placental growth factor, hepatocyte growth factor, platelet activating factor, insulin-like growth factor, interleukin-8, or granulocyte-colony stimulating factor.

57. The pharmaceutical composition of claim 45, further comprising a pharmaceutically acceptable carrier.

58. The pharmaceutical composition of claim 45 in which the subject is a human.

59. The pharmaceutical composition of claim 45 in which the angiogenesis-related disease is selected from the group consisting of fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma, acoustic neuroma, neurofibroma, trachoma and pyogenic granulomas.

60. The pharmaceutical composition of claim 59 wherein the angiogenesis-related disease is breast cancer.

61. The pharmaceutical composition of claim 59 wherein the angiogenesis-related disease is prostate cancer.

62. The pharmaceutical composition of claim 45 in which the angiogenesis-related disease is selected from the group consisting of acute lymphocytic leukemia and acute myelocytic leukemia, chronic leukemia, polycythemia vera, lymphoma, multiple myeloma, Waldenström's macroglobulinemia, and heavy chain disease.

63. The pharmaceutic composition of claim 45, wherein the anti-neoplastic agent is selected from the group consisting of docetaxel, etoposide, trontecan,

paclitaxel, teniposide, topotecan, vinblastine, vincristine, and vindesine, busulfan, improsulfan, piposulfan, aziridines, benzodepa, carboquone, meturedepa, uredepa, altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide, chlorambucil, chloraphazine, cyclophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, perfosfamide, phenesterine, prednimustine, trofosfamide, uracil mustard, carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine, dacarbazine, mannomustine, mitobronitol, mitolactol, pipobroman, temozolomide, aclacinomycinsa actinomycin F_1, anthramycin, azaserine, bleomycins, cactinomycin, carubicin, carzinophilin, chromomycins, dactinomycin, daunorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, idarubicin, menogaril, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, pirarubicin, plicamycin, porfiromycin, puromycin, streptonigrin, streptozocin, tubercidin, zinostatin, zorubicin, denopterin, edatrexate, methotrexate, piritrexim, pteropterin, Tomudex®, trimetrexate, cladribine, fludarabine, 6-mercaptopurine, thiamiprine, thioguanine, ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, doxifluridine, emitefur, enocitabune, floxuridine, fluorouracil, gemcitabine, tegafur, L-asparaginase, interferon-α, interferon-β, interferon-γ, interleukin-2, lentinan, propagermanium, PSK, roquinimex, sizofican, ubenimex, carboplatin, cisplatin, miboplatin, oxaliplatin, aceglarone, amsacrine, bisantrene, defosfamide, demecolcine, diaziquone, eflornithine, elliptinium acetate, etoglucid, fenretinide, gallium nitrate, hydroxyurea, lonidamine, miltefosine, mitoguazone, mitoxantrone, mopidamol, nitracine, pentostain, phenamet, podophyllinic acid 2-ethyl-hydrazide, procabazine, razoxane, sobuzoxane, spirogermanium, tenuzonic acid, triaziquone, 2,2',2"trichlorotriethylamine, urethan, calusterone, dromostanolone, epitiostanol, mepitiostane, testolacone, aminoglutethimide, mitotane, trilostane, bicalutamide, flutamide, nilutamide, droloxifene, tamoxifen, toremifene, aminoglutethimide, anastrozole, fadrozole, formestane, letrozole, fosfestrol, hexestrol, polyestradiol phosphate, buserelin, goserelin, leuprolide, triptorelin, chlormadinone acetate, medroxyprogesterone, megestrol acetate, melengestrol, porfimer sodium, batimastar, and folinic acid.

64. A method for selecting a neomycin analogue for use in inhibiting angiogenesis or proliferation of endothelial cells, comprising testing the neomycin analogue for activity for inhibiting angiogenesis.

65. The method according to claim 64, which comprises

(a) incubating a first culture of endothelial cells with the neomycin analogue and an angiogenic factor in a growth medium, and incubating a second culture of endothelial cells with the angiogenic factor in the growth medium lacking the neomycin analogue, wherein the angiogenic factor is labeled;

(b) determining the amounts of angiogenic factor present in the nuclei of cells in the first and the second cultures; and

(c) selecting for use in treating the angiogenesis-related disease, the neomycin analogue that inhibits nuclear translocation of the angiogenic factor in cells of the first culture by at least 10% of the amount of the angiogenic factor translocated to the nuclei of the cells in the second culture.

66. The method according to claim 64, which comprises

(a) incubating a first culture of endothelial cells with the neomycin analogue in a growth medium, and incubating a second culture of endothelial cells in a growth medium lacking the neomycin analogue;

(b) incubating the first and the second cultures with an angiogenic factor in the growth medium, wherein the angiogenic factor is labeled;

(c) determining the amount of angiogenic factor present in the nuclei of cells in the first and the second cultures; and

(d) selecting for use in treating the angiogenesis-related disease, the neomycin analogue that inhibits nuclear translocation of the angiogenic factor in the cells of the first culture by at least 10% of the amount of nuclear translocation of the angiogenic factor in the cells of the second culture.

67. The method according to claim 64, which comprises

(a) incubating a first culture of endothelial cells with the neomycin analogue and an angiogenic factor in a growth medium, incubating a second culture of endothelial cells with the neomycin analogue in the growth medium lacking the angiogenic factor, incubating a third culture of endothelial cells with the angiogenic factor in the growth medium lacking the neomycin analogue, incubating a fourth culture of endothelial cells in the growth medium lacking the neomycin analogue and the angiogenic factor;

(b) determining the cell numbers of the first, the second, the third and the fourth cultures; and

(c) selecting for use in treating the angiogenesis-related disease, the neomycin analogue that reduces the increase in the cell number in the second culture over the cell number in the first culture to less than about 75% of the increase in cell number of the third culture over the cell number of the fourth culture.

68. The method according to claim 64, which comprises

(a) contacting the chorioallantoic membrane of a first group of chick embryos with the neomycin analogue and an angiogenic factor, contacting the chorioallantoic membrane of a second group of chick embryos with the neomycin analogue but not the angiogenic factor, contacting the chorioallantoic membrane of a third group of chick embryos with the angiogenic factor but not the neomycin analogue, and contacting the chorioallantoic membrane of a fourth group of chick embryos with a solution lacking the neomycin analogue and the antigenic factor;

(b) incubating the first, the second, the third and the fourth groups of chick embryos;

(c) determining the numbers of embryos having an angiogenic response in the first, the second, the third and the fourth groups of embryos; and

(d) selecting for use in treating the angiogenesis-related disease, the neomycin analogue that reduces the increase in the number of embryos exhibiting an angiogenic response in the second group of embryos over the number of embryos exhibiting an angiogenic response in the first group of embryos to less than about 75% of the increase in the number of embryos exhibiting an angiogenic response in the third group of embryos over the number of embryos exhibiting an angiogenic response in the fourth group of embryos.

69. The method according to any one of claims 64 to 68, wherein the neomycin analogue is

(a) neomycin A, neomycin B, or neomycin C;

(b) a complex comprising neomycin A, neomycin B, or neomycin C;

(c) an aminoglycoside having a structure substantially similar to that of neomycin A, neomycin B or neomycin C;

(d) a chemical or biological breakdown product of neomycin A, neomycin B or neomycin C;

(e) a derivative of neomycin A, neomycin B or neomycin C; or

(f) a naturally-occurring precursor to neomycin A, neomycin B or neomycin C.

70. The method according to claim 69, wherein the neomycin analogue comprises a substituted-2-deoxystreptamine (2-DOS) linked to two to four sugars, wherein each sugar is a pentose or hexose.

71. The method according to claim 70, wherein the neomycin analogue is a member of the neomycin, paromomycin or lividomycin aminoglycoside family.

72. The method according to claim 71, wherein the neomycin analogue comprises a glucosyl residue attached to the 4 position of the 2-DOS moiety, which glucosyl residue comprises an amino group at each of the 2 and 6 positions.

73. The method according to claim 72, wherein the neomycin analogue comprises a 2-DOS and a 2,6-diamino-2-6-dideoxy-D-glucose attached to the 4 position of 2-DOS

74. The method according to any one of claims 64 to 68, wherein the angiogenic factor is an acidic fibroblast growth factor, basic fibroblast growth factor, epidermal growth factor, tumor growth factor-alpha, tumor growth factor-beta, tumor necrosis factor-alpha, vascular endothelial growth factor, platelet-derived growth factor, platelet-derived endothelial cell growth fqactor, placental growth factor, hepatocyte growth fctor, platelet activating factor, insulin-like growth factor, interleukin-8, or granulocyte-colony stimulating factor.

75. The method according to any one of claims 64 to 68, in which the angiogenesis or proliferation of endothelial cells is associated with a disease selected from the group consisting of fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma, acoustic neuroma, neurofibroma, trachoma and pyogenic granulomas.

76. The method according to any one of claims 64 to 68 wherein the disease is breast cancer.

77. The method according to any one of claims 64 to 68 wherein the disease is prostate cancer.

78. The method according to any one of claims 64 to 68, in which the angiogenesis or proliferation of endothelial cells is associated with a disease selected from the group consisting of acute lymphocytic leukemia and acute myelocytic leukemia, chronic leukemia, polycythemia vera, lymphoma, multiple myeloma, Waldenström's macroglobulinemia, and heavy chain disease.

79. The method according to any one of claims 64 to 68, in which the angiogenesis or proliferation of endothelial cells is associated with a disease selected from the group consisting of acne rosacea, atopic keratitis, chemical burns, contact lens overwear, corneal graft rejection, diabetic retinopathy, epidemic keratoconjunctivitis, fungal ulcers, Herpes simplex infections, Herpes zoster infections, Kaposi sarcoma, lipid degeneration, marginal keratolysis, Mooren ulcer, neovascular glaucoma and retrolental fibroplasia, periphigoid radial keratotomy, phylectenulosis, polyarteritis, protozoan infections, pterygium keratitis sicca, retinopathy of prematurity, rheumatoid arthritis, sjogrens, scleritis, Steven's Johnson disease, superior limbic keratitis, syphilis, systemic lupus, Terrien's marginal degeneration, trauma, Vitamin A deficiency, and Wegeners sarcoidosis.

80. The method according to any one of claims 64 to 68, in which the angiogenesis or proliferation of endothelial cells is associated with a disease selected from the group consisting of artery occlusion, Bechets disease, Bests disease, chronic retinal detachment, chronic uveitis/vitritis, carotid obstructive disease, diabetic retinopathy, Eales disease, hyperviscosity syndromes, retinitis, choroiditis, Lyme's disease, macular degeneration, optic pits, Pagets disease, pars planitis, post-laser complications, presumed ocular histoplasmosis, pseudoxanthoma elasticum,

retinopathy of prematurity, sickle cell anemia, sarcoid, Stargarts disease, syphilis, systemic lupus erythematosis, toxoplasmosis, trauma, vein occlusion, rubeosis, and proliferative vitreoretinopathy.

81. The method according to any one of claims 64 to 68, in which the angiogenesis or proliferation of endothelial cells is associated with a disease selected from the group consisting of Crohn's disease and ulcerative colitis, psoriasis, rheumatoid arthritis, sarcoidosis, hemangiomas, Osler-Weber-Rendu disease, hereditary hemorrhagic telangiectasia, and acquired immune deficiency syndrome.

state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. Such pharmaceutically acceptable carriers or diluents and methods for preparing are well known in the art (see, *e.g.*, Remington's Pharmaceutical Sciences, Meade Publishing Col., Easton, PA, latest edition; the Handbook of Pharmaceutical Excipients, APhA publications, 1986).

Pharmaceutically acceptable carriers may be, for example, a liquid or solid. Liquid carriers include, but are not limited, to water, saline, buffered saline, dextrose solution, preferably such physiologically compatible buffers as Hank's or Ringer's solution, physiological saline, a mixture consisting of saline and glucose, and heparinized sodium-citrate-citric acid-dextrose solution and the like, preferably in sterile form. Exemplary solid carrier include agar, acacia, gelatin, lactose, magnesium stearate, pectin, talc and like.

Compositions of the invention can be administered orally. For such administrations, the pharmaceutical composition may be in liquid form, for example, solutions, syrups or suspensions, or may be presented as a drug product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats or oils); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, or fractionated vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The pharmaceutical compositions may take the form of, for example, tablets, capsules or pellets prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.*, pregelatinized maize starch, polyvinyl pyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (*e.g.*, magnesium stearate, talc or silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets may be coated by methods well-known in the art.

For buccal administration, the compositions may take the form of tablets, troche or lozenge formulated in conventional manner.

Compositions, *e.g.*, for oral or buccal administration, may be suitably formulated to give controlled release of the active compound. Such formulations may include one or

more sustained-release agents known in the art, such as glyceryl mono-stearate, glyceryl distearate and wax.

Compositions of the invention may be applied topically. Such administrations includes applying the compositions externally to the epidermis, the mouth cavity, and the instillation into the eye, ear and nose, such that the neomycin or Analogue does not significantly enter the blood stream. This contrasts with systemic administration achieved by oral, intravenous, intraperitoneal and intramuscular delivery.

Compositions for use in topical administration include, *e.g.*, liquid or gel preparations suitable for penetration through the skin such as creams, liniments, lotions, ointments or pastes, and drops suitable for delivery to the eye, ear or nose.

According to the invention, creams, drops, liniments, lotions, ointments and pastes are liquid or semi-solid compositions for external application. Such compositions may be prepared by mixing the active ingredient(s) in powdered form, alone or in solution or suspension in an aqueous or non-aqueous fluid with a greasy or non-greasy base. The base may comprise complex hydrocarbons such as glycerol, various forms of paraffin, beeswax; a mucilage; a mineral or edible oil or fatty acids; or a macrogel. Such compositions may additionally comprise suitable surface active agents such as surfactants, and suspending agents such as agar, vegetable gums, cellulose derivatives, and other ingredients such as preservatives, antioxidants, etc.

According to the invention, lotions and drops include those suitable for application to the eye or skin. Eye lotions and drops may comprise a sterile aqueous solution, oily solutions or suspensions maybe prepared by dissolving the active ingredient(s) in a suitable aqueous solution. Such solutions may optionally contain a suitable bactericide, fungicide, preservative, and surfactant. Lotions or liniments for applying to the skin may also comprise drying agents such as alcohol and/or a moisturizer such as glycerol, an oil or fatty acid.

Compositions of the invention also can be administered nasally or by inhalation. For nasal or inhalation administration, the compositions are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane,

dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

Compositions of the invention may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example, subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophilic drugs.

Compositions of the invention comprise neomycin or Analogue, which may be in the form of a free base or acid, or a pharmaceutically acceptable salt thereof. Such salts are well known in the art. They include, but are not limited to, salts of inorganic and organic acids such as hydrochloric acid, hydrobromic acid, phosphoric acid, sulfuric acid, acetic acid, citric acid, fumaric acid, lactic acid, maleic acid, oxalic acid, phenylacetic acid, salicylic acid, succinic acid, and tartaric acid.

In preferred embodiments, compositions of the invention comprise an active ingredient (*i.e.*, neomycin, Analogues, anti-angiogenic agents, and anti-neoplastic agents) that is a purified preparation.

Techniques and formulations for administering above-described compositions may be found in Remington's Pharmaceutical Sciences, Meade Publishing Col., Easton, PA, latest edition.

5.3. ADMINISTRATION OF NEOMYCIN OR ANALOGUE

The present invention contemplates administration of pharmaceutical compositions comprising neomycin or analogue thereof to (a) inhibit the pathological angiogenesis associated with an angiogenesis-related disease, or (b) ameliorate or eliminate any other pathological symptoms of the disease. The dose of the neomycin or Analogue to be

administered is a therapeutic amount effective to inhibit the formation or spread of inappropriate, undesired or excessive blood vessels at the disease site, *e.g.*, as detected by such ability *in vivo*, or as extrapolated from *in vitro* assays (*e.g.*, an assay that determines activity in inactivating or inhibiting the angiogenic factor-induced proliferation of endothelial cells) or from an animal model system such as the CAM assay or the corneal neovascularization assay. According to the invention, neomycin or Analogue may be administered in a single dose, or sustained administration, *e.g.*, by intravenous (IV) drip or pump, or multiple doses.

Where the administration is in form of multiple doses, it should be at a frequency that is effective to inhibit the formation or spread of inappropriate, undesired or excessive blood vessels at the disease site, *e.g.*, as detected by such ability *in vivo*, or as extrapolated from *in vitro* assays (*e.g.*, an assay that determines activity in inactivating or inhibiting the angiogenic factor-induced proliferation of endothelial cells) or from an animal model system such as the CAM assay or the corneal neovascularization assay.

The present invention contemplates a daily dosage of neomycin or Analogue from about 0.5 μg/kg body weight/day to about 0.1 gm/kg body weight/day when the composition of the invention is administered orally, and from about 0.5 μg/kg body weight/day to about 0.06 gm/kg body weight/day when the composition is administered parenterally.

Where the subject being treated is human, in one embodiment of the present invention, neomycin is administered orally to the subject in divided doses totalling from about 4 Gm to about 8 Gm per day; in another embodiment, neomycin is administered intramuscularly to the subject using a daily dose of about 1 to about 6 Gm; in another embodiment, neomycin is administered parenterally to the subject using a dosage of 6 Gm or less.

The schedule of the neomycin or Analogue treatment should be at a periodicity that is sufficient to inhibit the formation or spread of inappropriate, undesired or excessive blood vessels at the disease site, and allows the subject to partially or completely recover from any undesirable side-effects caused or contributed to by the neomycin or Analogue treatment.

The duration of the neomycin or Analogue treatment should be for the length of time sufficient to inhibit the formation or spread of inappropriate, undesired or excessive blood vessels at the disease site, or preferably to cure the angiogenesis-related disease. The present invention contemplates a duration of treatment from one day up to several months.

The choice of the particular composition, form for administration, and effective dosages, as well as the frequency, schedule and duration of treatment will vary depending in part on the angiogenesis-related disease being treated.

5.4. ANGIOGENESIS-RELATED DISEASES

The present invention provides method for treating or curing angiogenesis-related diseases, which involve excessive, inappropriate or undesired angiogenesis (*i.e.*, pathological angiogenesis). Angiogenesis-related diseases may also involve excessive, inappropriate or undesired proliferation and/or migration of endothelial cells. Many diseases are associated with, or based on pathological angiogenesis or proliferation of endothelial cells. Angiogenesis-related diseases are myriad and varied. They include, but are not limited to, various forms of neovacularization or hypervascularization diseases, inflammatory diseases, arthritis and cancer.

As contemplated by the present invention, many solid and blood-borne tumors are angiogenesis-related diseases and are susceptible to treatment by the method of the invention. Solid tumors that may be treated by the method of the invention include, but are not limited to sarcomas and carcinomas, *e.g.*, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma,

astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma, and benign solid tumors such as acoustic neuroma, neurofibroma, trachoma and pyogenic granulomas.

Blood-borne tumors such as leukemias that are susceptible to treatment by the method of the invention include, but are not limited to, acute lymphocytic leukemia and acute myelocytic leukemia (myeloblastic, promyelocytic, myelomonocytic, monocytic and erythroleukemia); chronic leukemia (chronic myelocytic [granulocytic] leukemia and chronic lymphocytic leukemia); and polycythemia vera, lymphoma (Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenström's macroglobulinemia, and heavy chain disease.

Many corneal diseases involve pathological neovascularization, and hence are angiogenesis-related diseases and susceptible to treatment by the method of the invention. Such corneal neovascularization diseases include, but are not limited to, acne rosacea, atopic keratitis, bacterial ulcers, chemical burns, contact lens overwear, corneal graft rejection, diabetic retinopathy, epidemic keratoconjunctivitis, fungal ulcers, Herpes simplex infections, Herpes zoster infections, Kaposi sarcoma, lipid degeneration, marginal keratolysis, mycobacteria infections, Mooren ulcer, neovascular glaucoma and retrolental fibroplasia, periphigoid radial keratotomy, phylectenulosis, polyarteritis, protozoan infections, pterygium keratitis sicca, retinopathy of prematurity, rheumatoid arthritis, sjogrens, scleritis, Steven's Johnson disease, superior limbic keratitis, syphilis, systemic lupus, Terrien's marginal degeneration, trauma, Vitamin A deficiency, and Wegeners sarcoidosis.

Similarly, many retinal/corneal diseases also involve pathological neovascularization, and thus are also angiogenesis-related diseases that are susceptible to treatment by the method of the invention. Such diseases include, but are not limited to, artery occlusion, Bechets disease, Bests disease, chronic retinal detachment, chronic uveitis/vitritis, carotid obstructive disease, diabetic retinopathy, Eales disease, hyperviscosity syndromes, infections causing a retinitis or choroiditis, Lyme's disease, macular degeneration, mycobacterial infections, optic pits, Pagets disease, pars planitis,

post-laser complications, presumed ocular histoplasmosis, pseudoxanthoma elasticum, retinopathy of prematurity, sickle cell anemia, sarcoid, Stargarts disease, syphilis, systemic lupus erythematosis, toxoplasmosis, trauma, and vein occlusion. Other such diseases include, but are not limited to, diseases associated with rubeosis and diseases caused by the abnormal proliferation of fibrovascular or fibrous tissue including all forms of proliferative vitreoretinopathy, whether or not associated with diabetes.

Many chronic inflammatory diseases also involve pathological angiogenesis, and thus can be treated by the method of the present invention. Such diseases include, but are not limited to, inflammatory bowel diseases such as Crohn's disease and ulcerative colitis, psoriasis, rheumatoid arthritis, and sarcoidosis.

Other diseases that involve pathological angiogenesis include hemangiomas, Osler-Weber-Rendu disease, or hereditary hemorrhagic telangiectasia, and acquired immune deficiency syndrome.

Accordingly, subjects having angiogenesis-related diseases would also benefit from therapeutic treatment with the method of the invention.

The invention can be better understood by referring to the following examples, which are provided merely by way of exemplification and are not intended to limit the invention.

6. EXAMPLES

6.1. NEOMYCIN INHIBITS ANGIOGENIN-INDUCED ANGIOGENESIS

This set of experiments demonstrates that the aminoglycoside antibiotic neomycin, a known PLC inhibitor, is a potent inhibitor of both nuclear translocation of angiogenin, as well as angiogenin-induced cell proliferation and angiogenesis. The results indicate that neomycin is a new type of anti-angiogenic agent that may serve in the clinical treatment of angiogenesis-related diseases.

6.1.1. MATERIALS AND METHODS

6.1.1.1. MATERIALS

Human angiogenin (Met-1) was a recombinant product from an *Escherichia coli* expression system (Shapiro et al., 1988, Anal. Biochem. 175:450-461). Fertilized chicken eggs were from Spafas. Neomycin, amikacin, gentamicin, kanamycin, paromomycin, streptomycin, penicillin, amoxicillin, bacitracin, erythromycin, staurosporine, oxophenylarsine, yeast tRNA, and ribonuclease-free BSA were from Sigma Chemicals Co; U-73122 and U-73343 were from CalBiochem; genistein was from ICC; basic fibroblast growth factor (bFGF) was from Promega; human endothelial serum-free medium (HE-SFM) was from GIBCO/BRL-Life Technologies; fetal bovine serum (FBS) was from Hyclone; excellulose GF-5 desalting columns and Iodo-Beads iodination reagents were from Pierce; methyl-[^{3}H]-thymidine (6.7 Ci/mmol, 1 Ci = 37 Gbq) and Na^{125}I (17.4 Ci/mg) were from Dupont/NEN.

6.1.1.2. CELL CULTURE

Human umbilical vein endothelial (HUVE) cells were purchased from Cell Systems Corp. (Kirkland, WA). The cells were cultured in HE-SFM supplemented with 10% FBS and 10 ng/ml bFGF at 37°C under 5% humidified CO_2 and were split 1:3 for subculture. Cells between passages 5 and 12 inclusive were used for all experiments. Cell numbers were determined with a Coulter counter, and cell viability was measured by trypan blue dye exclusion assay.

6.1.1.3. IODINATION OF ANGIOGENIN

^{125}I-labeled angiogenin was prepared with the use of Iodo-Beads as described previously (Hu et al., 1997, Proc. Natl. Acad. Sci. USA 94:2204-2209). The specific activity of ^{125}I-angiogenin used in the experiments ranged from 1-2 x 10^6 cpm/μg.

6.1.1.4. NUCLEAR TRANSLOCATION

HUVE cells were seeded at 5 x 10^3 cells/cm^2 in 35 mm dishes and cultured in HE-SFM supplemented with 20 ng/ml bFGF at 37°C under 5% humidified CO_2 for 24 hr. The

cells were washed three times with prewarmed (37°C) HE-SFM and incubated with ^{125}I-angiogenin (1 μg/ml) at 37°C for 30 min. Two procedures were used to examine the effect of inhibitors on nuclear translocation. The first was to premix the inhibitors with ^{125}I-angiogenin and adjust the sample volume to 10 μl with HE-SFM before addition to the cells. The second was to pretreat the cells in HE-SFM with the inhibitors for 10 to 30 min before ^{125}I-angiogenin was added to the cells. After incubation, the dishes were cooled at 4°C for 10 min and the medium was removed. The cells were washed three times with cold phosphate-buffered saline (PBS), detached by scraping, and centrifuged at 800 x g for 5 min. The cells were washed once with PBS and lysed by 0.5% Triton X-100 in PBS. The nuclear fraction was isolated by centrifugation at 1200 x g for 5 min. Radioactivity was determined with a gamma counter.

6.1.1.5. CELL PROLIFERATION

HUVE cells were seeded at 4 x 10^3 cells/cm^2 in attachment factor (Cell Systems Corp.)-coated 35 mm dishes in HE-SFM, and incubated with 1 μg/ml angiogenin in the presence or absence of inhibitors at 37°C for 48 hr. Cell were detached by trypsinization and cell numbers were determined with a Coulter counter.

6.1.1.6. RIBONUCLEOLYTIC ACTIVITY ASSAY

The effect of neomycin on the ribonucleolytic activity of angiogenin was examined with yeast tRNA as the substrate. Angiogenin, or its mixture with neomycin was added to an assay mixture containing 0.6 mg of yeast tRNA, 30 μg of ribonuclease-free BSA, 30 mM HEPES, pH 6.8, and 30 mM NaCl in a final volume of 300 μl. After incubation for 2 hr at 37°C, 700 pl of 3.4% ice-cold perchloric acid was added, the mixture was vortexed, kept on ice for 10 mm and centrifuged at 15,000 x g for 10 min at 4°C. The absorbance of the supernatants was measured at 260 nm.

6.1.1.7. ANGIOGENESIS ASSAY

Angiogenesis was measured on the CAM by the method of Knighton *et al.* (Knighton et al., 1977, Br. J. Cancer 35:347-356) essentially as described (Fett et al., 1985,

Biochemistry 24:5480-5486). Fertilized chicken eggs were kept at 18°C for 2 days and then incubated in a humidified environment at 37°C for 3 days. Albumin was aspirated from the embryos and after 24 hours, a "window" was cut through the shell and covered with clear tape. The embryos were incubated for another 6 days at 37°C before an angiogenic factor and/or neomycin were applied. The angiogenic factor and/or neomycin each in about 5 µl of H_2O were applied to sterile, Thermanox 15-mm disks, dried under laminar flow, and applied to the CAM surface sample side down. After 48-68 hours at 37°C, the growth of blood vessels was observed microscopically and recorded as either positive or negative. A positive response (*i.e.*, an angiogenic response) has a typical "spokewheel" appearance.

6.1.2. RESULTS

6.1.2.1. NEOMYCIN INHIBITS NUCLEAR TRANSLOCATION OF ANGIOGENIN

Exogenously added angiogenin is rapidly taken up and translocated to the nucleus of proliferating endothelial cells (Moroianu et al., 1994, Proc. Natl. Acad Sci. USA 91:1677-1681). The mechanism of translocation is not yet known; but it seems to be energy and temperature dependent, suggesting the involvement of receptor-mediated endocytosis (Moroianu et al., 1994, Proc. Natl. Acad Sci. USA 91:1677-1681). Angiogenin also induces DNA synthesis and cell proliferation of sparsely cultured human endothelial cells (Hu et al., 1997, Proc. Natl. Acad. Sci. USA 94:2204-2209). Accordingly, the relationship of signal transduction and nuclear translocation was investigated by examining the effect of specific inhibitors of enzymes thought to be involved in the signal transduction process on the nuclear translocation of angiogenin in HUVE cells. As shown in Table 1, genistein and oxophenylarsine, inhibitors of tyrosine kinase and phosphotyrosine phosphatase (Mayer et al., 1995, J. Pharm. Exp. Therap. 274:427-436), respectively, have no effect on nuclear translocation of ^{125}I-angiogenin. Staurosporine, an inhibitor of protein kinase C, at its optimal concentration of 100 nM (Mayer et al., 1995, J. Pharm. Exp. Therap. 274:427-436), was only marginally inhibitory. However, 100 µM neomycin, an aminoglycoside antibiotic and a PLC inhibitor (Somjen et al., 1997, J. Cell. Biochem. 65:53-66, Hildebrandt et al.,

1997, Bri. J. Phar. 120:841-8S0), decreased the amount of ^{125}I-angiogenin accumulated in the cell nucleus after 30 min incubation by up to 60%. Another inhibitor of PLC, U-73122, also showed significant inhibition of nuclear translocation of ^{125}I-angiogenin (30% inhibition at 10 μM), whereas, its inactive analogue, U-73343, had no effect. These data indicate that inhibitors of PLC inhibit nuclear translocation of angiogenin in HUVE cells, implying that PLC activity is required for translocation.

Table 1. Inhibition of Nuclear Translocation of Angiogenin

Inhibitors	Nuclear ^{125}I-angiogenin (cpm)	% inhibition
Control	3090 ± 260	0
Genistein (100 μM)	3300 ± 170	0
Oxophenylarsine (10 μM)	3040 ± 70	0
Staurosporine (100 nM)	2710 ± 70	12
Neomycin (100 μM)	1230 ± 60	60
U-73122 (10 μM)	2140 ± 30	31
U-73343 (10 μM)	2890 ± 100	6

HUVE cells, 50,000 per 35 mm dish, were treated with inhibitors at 37°C for 30 min ^{125}I-angiogenin was added to a final concentration of 1 μg/ml and incubated at 37°C for 30 min. Nuclear fractions were isolated and radioactivities determined.

Neomycin inhibits nuclear translocation of angiogenin in a dose-dependent manner (Fig. 1). Increasing concentration of neomycin progressively decrease the amount of nuclear accumulated ^{125}I-angiogenin from 3090 ± 260 cpm in the control to 420 ± 100 cpm in the presence of 500 μM inhibitor. The inhibition is not linear. At 10 μM, nuclear translocation is already inhibited by 42%. Increasing the concentration to 200 μM only increases inhibition by another 23%. Nuclear translocation cannot be completely abolished

by neomycin. At 500 μM, the amount of ^{125}I-angiogenin that accumulates in the nucleus is 14% of that in the control.

6.1.2.2. NEOMYCIN INHIBITS ANGIOGENIN-INDUCED CELL PROLIFERATION

Exogenous angiogenin stimulates DNA synthesis and cell proliferation of sparsely cultured human endothelial cells (Hu et al., 1997, Proc. Natl. Acad. Sci. USA 94:2204-2209). Since neomycin inhibits nuclear translocation of angiogenin, the inhibitor's effect on angiogenin-induced cell proliferation was examined. When cells were cultured under the conditions described, essentially all were recovered after 48 hr in the absence of angiogenin and neomycin. In the presence of 1 μg/ml angiogenin, cell number after 48 hr increased by 35%. Neomycin alone neither induced nor inhibited cell proliferation. However, it inhibited angiogenin-induced cell proliferation in a dose-dependent but non-linear manner. Thus, 5 μM neomycin already inhibited the proliferative activity of angiogenin by 49% (Fig. 2). Increasing the neomycin concentration to 25 μM inhibited angiogenin-induced cell proliferation by 69% and at 50 μM, it was completely abolished.

6.1.2.3. NEOMYCIN INHIBITS ANGIOGENIN-INDUCED ANGIOGENESIS

The ability of neomycin to inhibit angiogenin-induced angiogenesis was tested in the CAM assay. As shown in Table 2, neomycin itself at the concentration ranging from about 5 to about 50 μM (20 to 200 ng in the 5 μl volume applied) does not induce angiogenesis, nor does it cause necrosis or any other visible adverse effect on the chick embryo. Angiogenin alone at 10 ng induced a positive response in 55% of the chick embryos, consistent with previous results (Fett et al., 1985, Biochemistry 24:5480-5486). Neomycin at 4 ng decreased the number of angiogenic responses induced by 10 ng angiogenin from 55% to 40%, and at 20 ng decreased it to 20%, the same percentage obtained with water control. Thus, a dose of 20 ng neomycin/embryo or higher completely inhibits angiogenin-induced angiogenesis.

Table 2. Effect of Neomycin on the Activity of Angiogenin in the CAM Assay

Samples	Total Embryos	% Positive
Angiogenin (10 ng)	76	55
Neomycin (20 ng)	50	20
Neomycin (200 ng)	29	21
Angiogenin (10 ng)		
+ Neomycin (4 ng)	40	40
Angiogenin (10 ng)		
+ Neomycin (20 ng)	40	20
Angiogenin (10 ng)		
+ Neomycin (200 ng)	20	25
Water	128	20

Data were combined from multiple sets of experiments each using between 10 and 20 embryos.

6.1.2.4. NEOMYCIN'S EFFECT ON THE RIBONUCLEOLYTIC ACTIVITY OF ANGIOGENIN

The effect of neomycin on the ribonucleolytic activity of angiogenin was examined with yeast tRNA as the substrate. The ribonucleolytic activity of angiogenin in the presence of 5 μM, 10 μM, and 50 μM neomycin was 87%, 105% and 88% of that of the control. At higher concentrations, neomycin forms precipitates with tRNA. These results show that neomycin does not inhibit the cleavage of yeast tRNA by angiogenin even at a concentration of 50 μM when the proliferative and angiogenic activities were already completely abolished. These data suggest that the inhibitory activity of neomycin on angiogenin-induced blood vessel formation is not attributable to its effect on the

ribonucleolytic activity of angiogenin, but rather to its inhibition of nuclear translocation of angiogenin in endothelial cells and/or its inhibition of angiogenin-induced cell proliferation.

6.1.2.5. EFFECTS OF OTHER AMINOGLYCOSIDE ANTIBIOTICS ON ANGIOGENIN-INDUCED CELL PROLIFERATION OR ANGIOGENESIS

Other members of aminoglycoside antibiotic family were also examined for their ability to inhibit angiogenin-induced proliferation of endothelial cells. None of the commonly used aminoglycosides - streptomycin, kanamycin, gentamicin and amikacin - inhibited angiogenin-induced cell proliferation (Table 3). Significantly, paromomycin, which differs from neomycin only at position 6 of the glucose ring, did not inhibit angiogenin-induced cell proliferation. Thus, a single substitution of $-NH_2$ by -OH renders the aminoglycoside completely inactive as an anti-angiogenin agent. Data from CAM assay indicate that amikacin and streptomycin do not inhibit angiogenin-induced angiogenesis.

Table 3. Effects of Aminoglycoside Antibiotics on Angiogenin-induced Cell Proliferation

Aminoglycosides (100 μM)	Angiogenin (1 μg/ml)	Cell number	%*
None	-	52,000 ± 100	
	+	62,500 ± 100	120
Neomycin	-	52,700 ± 700	
	+	53,400 ± 1,900	101
Amikacin	-	51,700 ± 200	
	+	61,000 ± 400	118
Streptomycin	-	51,900 ± 1,300	
	+	59,900 ± 900	115
Kanamycin	-	48,800 ± 400	
	+	58,900 ± 200	121
Gentamicin	-	45,700 ± 500	
	+	55,700 ± 900	121
Paromomycin	-	50,900 ± 500	
	+	58,900 ± 400	116

* percent of cell number in the presence of 1 μg/ml angiogenin relative to the corresponding control.

6.1.3. DISCUSSION

Neomycin, an aminoglycoside, is an antibiotic that inhibits translation by binding to the small subunit of prokaryotic ribosomes causing misreading of mRNA. Unlike its structurally related compound, geneticin (G-418), which is known to bind the 80S ribosomes and block protein synthesis in eukaryotic cells and is therefore useful as a selective marker for gene transfection in eukaryotic cells (Southern et al., 1982, J. Mol. Appl. Genet. 1:327-341), neomycin does not bind to eukaryotic ribosomes. Neomycin up to

200 μM exhibited no cytotoxicity against HUVE cells. The cytotoxicity of other members of the aminoglycoside antibiotic family have also been examined. Such other aminoglycoside antibiotics, including amikacin, streptomycin, kanamycin, gentamicin and paromomycin, also exhibited no cytotoxicity against HUVE cells.

Among these aminoglycoside antibiotics, neomycin is the only one which shows inhibitory activity to angiogenin-induced cell proliferation. It is noteworthy that the structurally very similar aminoglycoside, paromomycin, has no inhibitory activity at all. Thus, the amino group on the carbon 6 of the glucose ring of neomycin apparently plays an important role in its inhibition of angiogenin-induced cell proliferation and angiogenesis.

Inhibition of nuclear translocation of angiogenin by neomycin is at least one of the reasons which lead to the inhibition of angiogenin-induced cell proliferation and angiogenesis. The concentrations required to inhibit nuclear translocation and cell proliferation by 50% are about 50 μM and 10 μM, respectively. Therefore, it is possible that some other functional aspects of neomycin, which remain to be investigated, may also contribute to its anti-angiogenesis activity.

Nuclear translocation of angiogenin in endothelial cells is thought to involve receptor-mediated endocytosis (Moroianu et al., 1994, Proc. Natl. Acad Sci. USA 91:1677-1681). However, binding of angiogenin to its surface receptor and the subsequent internalization do not seem to be inhibited by neomycin. Actually, neomycin induces a concomitant increase of cytosolic ^{125}I-angiogenin with the decrease of nuclear ^{125}I-angiogenin. If the PLC-inhibiting activity of neomycin is responsible for the inhibition of nuclear translocation of angiogenin, these results suggest that PLC activity is required for the steps subsequent to internalization in the nuclear translocation process. Since angiogenin activates PLC activity in endothelial cells (Bicknell et al., 1988, Proc. Natl. Acad Sci. USA 85:5961-5965) and PLC activity in turn is needed for nuclear translocation, the two cellular events may be interrelated and coordinate to function for the ultimate activity of angiogenin in endothelial cells. It is known that several cellular signal pathways activated by ligands binding to their receptors often crosstalk to obtain optimal cellular function (Jans, D. A., 1994, FASEB J. 8:841-847; Hopkins, C. R., 1994, Biochem. Pharma. 47:151-154).

Genistein, oxophenylarsine and staurosporine, which are inhibitors of tyrosine kinase, phosphotyrosine phosphatase and protein kinase C, respectively, do not inhibit nuclear translocation of angiogenin. It is unknown at present whether or not they inhibit angiogenin-induced proliferation and angiogenesis. If they do, the mechanisms would be different from that by which neomycin exerts its anti-angiogenesis effects.

The results disclosed here indicate that neomycin inhibits angiogenin-induced angiogenesis, mainly through its inhibition of nuclear translocation of angiogenin in endothelial cells. The data demonstrates that neomycin and its analogues are a new class of compounds having therapeutic use for treating angiogenesis-related diseases.

6.2. NEOMYCIN INHIBITS NUCLEAR TRANSLOCATION OF OTHER ANGIOGENIC FACTORS

The following experiments demonstrate that neomycin inhibits nuclear translocation of angiogenic factors other than angiogenin.

6.2.1. METHODS

Inhibition of nuclear translocation of angiogenic factors in HUVE cells was performed in the following manner. HUVE cells, passage 9 to 12, were cultured at 50,000 cells per 35 mm dish in HE-SFM supplemented with 20 ng/ml bFGF at 37°C for 24 hr. The cells were washed 3 times with prewarmed HE-SFM and treated with neomycin at various concentrations at 37°C for 10 min. ^{125}I-bFGF, ^{125}I-aFGF or ^{125}I-EGF, 50 ng/ml, was added and incubated at 37°C for 30 min. At the end of incubation, the cells were cooled at 4°C for 10 min and washed 3 times with cold PBS (4°C), detached by scraping and centrifuged at 800 x g for 5 min. The cell pellet was washed once with PBS and lysed with 0.5% triton X-100 in PBS. Nuclear fraction was isolated by centrifugation at 1200 x g for 5 min. Radioactivity in the nuclear fraction was determined with a gamma counter.

6.2.2. RESULTS

As shown in Table 4, neomycin inhibits nuclear translocation of bFGF, aFGF and EGF in HUVE cells in a dose- dependent manner. Neomycin's activity in inhibiting nuclear

translocation of these three angiogenic factors in HUVE cells is not as strong as its activity against the translocation of angiogenin (see Section 6.1.2.1, *supra*). At 10 μM, neomycin achieved 42% inhibition of the nuclear translocation of angiogenin, but only 13% and 15% inhibition of translocation of bFGF and aFGF, respectively. Nuclear translocation of EGF was not inhibited by neomycin until the latter's concentration exceeded 100 μM. However, since nuclear translocation of angiogenic proteins in endothelial cells is absolutely required for angiogenesis to occur, these lesser inhibitory activities are still sufficient in suppressing angiogenesis induced by these angiogenic factors (see Section 6.4, *infra*).

Table 4: Neomycin Inhibits Nuclear Translocation of FGFs and EGF

Neomycin (μM)	bFGF		aFGF		EGF	
	Counts (cpm)	% Inhib.	Counts (cpm)	% Inhib.	Counts (cpm)	% Inhib.
0	18300 ± 200	--	7800 ± 100	---	140 ± 20	--
10	15900 ± 100	13	6600 ± 100	15	140 ± 20	0
50	14300 ± 100	22	5800 ± 100	26	140 ± 20	0
100	13500 ± 300	26	5300 ± 100	32	130 ± 30	7
150	12400 ± 200	32	4800 ± 100	38	120 ± 10	14
200	10900 ± 100	40	4500 ± 200	43	100 ± 20	29

6.3. NEOMYCIN INHIBITS CELL PROLIFERATION INDUCED BY OTHER ANGIOGENIC FACTORS

These experiments demonstrate that neomycin inhibits cell proliferation induced by angiogenic factors other than angiogenin.

6.3.1. METHODS

Effect of neomycin on cell proliferation induced by angiogenic factors was performed in the following manner. HUVE cells, passage 8, were plated on attachment factor-coated 35 mm dish in HE-SFM at a density of 3000 cells/cm^2. bFGF (10 ng/ml), aFGF (10 ng/ml),

EGF (5 ng/ml) or VEGF (5 ng/ml) was added to the cells in the absence or presence of neomycin at different concentration immediately after the cells were seeded. The cells were incubated at 37°C under humidified air containing 5% CO_2 for 48 hrs. At the end of the incubation, the cells were washed once with PBS and detached by trypsinization. Cell numbers were determined with a Coulter counter.

6.3.2. RESULTS

As shown in Table 5, proliferation of HUVE cells induced by bFGF, aFGF and EGF was inhibited by neomycin in a dose- dependent manner. Thus, the proliferative activity of bFGF, aFGF and EGF was inhabited by 100 μM neomycin by 41%, 50% and 59%, respectively. As shown in Section 6.1.2.2., *supra*, neomycin inhibits angiogenin-induced proliferation of HUVE cells with an IC_{50} value of <10 μM. It appears that neomycin is a more potent and specific inhibitor for angiogenin than for the other angiogenic factors. VEGF is an angiogenic factor which has not been reported to undergo nuclear translocation in endothelial cells. Neomycin only has a small effect on VEGF-induced cell proliferation. Marginal inhibition (20%) was observed at 50 μM of neomycin. At 50 μM of neomycin, angiogenin-induced cell proliferation was already completely abolished. It is noteworthy that neomycin is not an effective inhibitor of cell proliferation induced by VEGF as it is of the proliferation induced by other angiogenic factors that have been tested. These results provides further evidence to support the hypothesis that neomycin inhibits angiogenesis, especially angiogenin-induced angiogenesis, via its inhibition of nuclear translocation of the angiogenic factors in endothelial cells.

Table 5: Inhibition of Cell Proliferation by Neomycin

Neomycin	Control	bFGF		aFGF		EGF		VEGF	
(μM)	Cell No.	Cell No.	Inhib. %	Cell No.	Inhib. %	Cell No.	Inhib. %	Cell No.	Inhib. %
0	31400±400	59600±2600	-	73900±2500	-	5300±300	-	45100±200	-
25	29800±300	51500±700	19	60000±400	25	46700±600	17	41000±300	14
50	28800±1000	45900±1200	34	51300±400	42	41700±600	35	38900±1300	20
100	27900±500	42600±200	41	46700±800	50	35700±1100	59	36400±400	22
150	27400±200	39900±125	49	41000±1000	63	32800±200	71	35000±200	36
200	26300±400	34600±400	64	37500±200	68	26000±900	100	33800±200	34

6.4. NEOMYCIN INHIBITS ANGIOGENESIS INDUCED BY OTHER ANGIOGENIC FACTORS

These experiments demonstrate that neomycin inhibits angiogenesis induced by other angiogenic factors.

6.4.1. METHODS

The ability of neomycin to inhibit bFGF-, CFGF-, EGF-, and VEGF-induced angiogenesis was tested in the CAM assay in a similar manner as described for angiogenin in Section 6.1.1.7, above.

6.4.2. RESULTS

As shown in Table 6, aFGF, bFGF, and EGF, at 10 ng per egg, induced angiogenesis in 73, 78, and 69% of the eggs, respectively. The percentages of positive eggs induced by the same concentration of these three angiogenic factors in the presence of 20 ng neomycin were 36, 45, and 60%, respectively, representing an inhibition of their angiogenic activity by 71, 58, and 19%, respectively. In the presence of 200 ng neomycin, the percentage of positive eggs were 32, 34, and 30%, not significantly different from that of the water control (21%) tested simultaneously. Neomycin did not significantly inhibit the angiogenic activity of VEGF. In the absence or presence of 200 ng and 1 μg neomycin, 10 ng of VEGF induced angiogenesis in 63, 58, and 52% of the eggs. Neomycin abolishes the angiogenic activity of angiogenin (10 ng) at a dose as low as 20 ng per egg (Section 6.1.2.3, above). Thus, neomycin inhibits angiogenesis induced by angiogenin, aFGF, bFGF and EGF, but not that stimulated by VEGF.

Table 6. Effect of neomycin on aFGF-, bFGF-, EGF- and VEGF-induced angiogenesis in CAM assay.

Sample	Neomycin (ng)	Total eggs	Positive eggs	% Positive	% Inhibition[a]
aFGF (10 ng)	0	49	36	73	-
"	20	14	5	36	71
"	200	47	16	34	75
bFGF (10 ng)	0	37	29	78	-
"	20	33	15	45	58
"	200	38	12	32	81
EGF (10 ng)	0	26	18	69	-
"	20	15	9	60	19
"	200	30	9	30	81
VEGF (10 ng)	0	27	17	63	-
"	200	24	14	58	10
"	1000	27	14	52	24
water[b]	0	212	45	21	-
	20	50	10	20	-
	200	29	6	21	-
	1000	13	3	23	-

Angiogenesis was measured on the chorioallantoic membrane as described above in Section 6.1.1.7. Growth of blood vessels was observed microscopically and recorded as either positive or negative after 48 hr of incubation. Data were combined from multiple sets of experiments each using between 10 and 20 eggs.

The angiogenic activity of VEGF in the chick CAM was not significantly inhibited by neomycin. The level of angiogenic response induced by 10 ng VEGF in the presence of 200 ng and 1 μg neomycin per embryo was 58% and 52%, respectively, not much different from that in the absence of neomycin (63%). These

data are in agreement with the results obtained in the proliferation assay where neomycin does not significantly inhibit VEGF-induced proliferation of HUVE cells.

VEGF is a pleiotropic angiogenic factor implicated in both developmental neovascularization and neoplastic angiogenesis, whereas the other angiogenic factors may be only related to disease status. Therefore, the fact that neomycin does not inhibit the angiogenic activity of VEGF may, on the one hand, reflect the finding that VEGF does not undergo nuclear translocation. On the other hand, it implies that neomycin may be a selective inhibitor of angiogenesis involved only in pathological conditions but not in the neovascularization under physiological circumstance. This is significant to the use of neomycin and its analogues as therapeutic agents for use in clinical treatment of angiogenesis-dependent disease. It indicates that the use of neomycin as an anti-angiogenic agent is specific and would not cause developmental abnormality. Neomycin, at 250 μM (1 μg in the 5 μl volume applied per embryo), did not cause necrosis or any other visible adverse effects on the chick embryo.

6.5. OTHER AMINOGLYCOSIDES DO NOT INHIBIT FGF-INDUCED CELL PROLIFERATIONS

These experiments demonstrate that other aminoglycoside antibiotics do not inhibit bFGF-induced proliferation of HUVE cells.

6.5.1. METHODS

The ability of the other members of the aminoglycoside antibiotic family to inhibit bFGF-induced proliferation of HUVE cells was examined in a similar manner as for angiogenin as described in Section 6.1.1.5, above. HUVE cells, passage 9, were seeded on attachment-factor coated dishes at 50,000 cells per 35 mm dish in HE-SFM. Aminoglycoside antibiotics were added and the cells were incubated with or without 10 ng/ml bFGF at 37°C for 48 hr.

6.5.2. RESULTS

As shown in Table 7, 100 μM neomycin inhibited bFGF-induced proliferation of HUVE cells by 71%. By contrast, no other members of the aminoglycoside antibiotic family tested, including streptomycin, kanamycin, gentamicin and amikacin, exhibited any significant inhibitory effect on cell proliferation induced by bFGF. These results are very similar to that obtained with angiogenin-induced cell proliferation presented in Section 6.1.2.5, *supra*. These data indicate that the anti-angiogenic and anti-bacterial activity of neomycin may depend on the different properties of the molecule and can be separated. It is known that the anti-bacterial function of neomycin and the other aminoglycoside antibodies is the result of binding to the 16S rRNA and inhibition of initiation of protein synthesis. The anti-angiogenic activity of neomycin may derive from its inhibition of PLC via binding to PIP_2, and the subsequent inhibition of nuclear translocation of angiogenic proteins. The lack of effect of other aminoglycoside antibiotics on the proliferative activity of angiogenin and bFGF further indicate that neomycin is a specific and selective inhibitor of angiogenesis.

Table 7: Effect of Aminoglycoside Antibiotics on bFGF-induced Cell Proliferation

Aminoglycoside (100μM)	Control	bFGF (10 ng/ml)	
		Cell Numbers	% Inhibition
Control	61300±500	99000±1000	-
Neomycin	58000±1500	68400±800	71
Streptomycin	62300±600	104200±400	0
Kanamycin	62600±1100	98000±2500	8
Gentamicin	61800±1200	98400±1900	5
Amikacin	63000±900	97600±1300	11

6.6. NEOMYCIN INHIBITS GROWTH OF PC-3 HUMAN PROSTATE TUMOR CELLS IN ATHYMIC MICE

These experiments established that neomycin inhibits the establishment and growth of PC-3 human prostate tumor cells inoculated in athymic mice.

6.6.1. METHODS

The subcutaneous tumor model in athymic mice has been used extensively to show that angiogenin antagonists such as monoclonal antibodies, its binding protein and antisense DNA, prevent the establishment of human tumor cells in mice (Olson et al., 1998, Proc. Am. Assoc. Cancer Res. 39:665A; Olson et al., 1994, Cancer Res. 54:4576-4579; Olson et al., 1995, Proc. Natl. Acad. Sci. USA 92:442-446. Olson KA et al., 1996, Proc. Am. Assoc. Cancer Res. 37:395A); none of these references, however, relate in any way to neomycin. As described below, this model is useful to examine the capacity of neomycin to delay or to prevent the establishment of PC-3 human prostate tumor cells in athymic mice.

Outbred athymic mice (6 mice per group) were injected subcutaneously with a mixture of 100 µl containing 1 x 10^4 PC-3 cells, 33 µl of basement membrane components (Matrigel), and either PBS control or neomycin at a dose of 20 mg/kg body weight. The mice received subcutaneous injections proximal to the site of the original cell inoculation of PBS control or neomycin diluted in PBS at a dose of 20 mg/kg body weight 6 times per week for 20 days, followed by injection 4 times per week for another 30 days. Mice were examined daily by palpation for the first sign of tumor appearance at which time tumor size was estimated twice weekly by caliper measurements (longest perpendicular length and width).

6.6.2. RESULTS

As shown in Fig. 3, treatment with neomycin prevented the appearance of PC-3 tumors in 50% of the mice. By day 18, 2 of 6 mice in the control group receiving PBS developed a tumor, whereas, all of the mice in the neomycin-treated group remained tumor-free. As of day 42, only 50% of the neomycin-treated as opposed to

100% of the animals in the control group had developed tumors. The dose (20 mg/kg body weight) used in this experiment was based on the usual intramuscular dose for human use (Wintrobe et al., 1971, Harrison's Principles of Internal Medicine, 6th ed., p749). There was no evidence of toxic side effects. No changes were observed between the control and neomycin-treated mice with respect to general activity, body weight, and food and fluid intake.

PC-3 cells are the most aggressive tumor cell line and are the least responsive one among the tumor cells so far tested for anti-angiogenin therapy. Thus, because neomycin is shown herein to be effective against PC-3 cells, it is expected to be more effective toward other tumor cells that are less aggressive than PC-3 cells.

6.7. NEOMYCIN INHIBITS ESTABLISHMENT AND GROWTH OF MDA-MB-435 HUMAN BREAST TUMOR CELLS IN ATHYMIC MICE

Theses experiments established that neomycin inhibits the establishment and growth of MDA-MB-435 human breast tumor cells inoculated in athymic mice.

6.7.1. METHODS

An orthotopic model was chosen to evaluate the efficacy of neomycin in preventing the growth of human breast cancer cells. MDA-MA-435 human breast tumor cells, which are estrogen receptor negative, were injected directly into the mammary fat pad of athymic mice. Age-matched athymic female mice were assigned to treatment groups of 8 mice each and anesthetized with ketamine (212 mg/kg body weight) and xylazine (21.2 mg/kg body weight) given intraperitoneally and allowed to stabilize under anesthesia for 15 min. A heating pad was used to maintain their body temperature throughout the procedure to minimize stress. Betadine followed by 70% alcohol was swabbed onto the skin of the left lateral thorax. An incision of 6 mm in length was made through the skin in the area of the left lateral thorax behind the left front leg and the mammary fat pad was exposed by using gentle pressure with two

fingers to separate the skin at the incision site. MDA-MB-435 human breast tumor cells were harvested by trypsinization, washed in HBSS, counted using trypan blue exclusion to determine cell viability, and 10,000 cells in a total volume of 20 µl were injected into the fat pad using a 27 gauge needle. The incision was closed with 2 drops of Vetbond veterinarian tissue adhesive and the mouse was allowed to recover on the heating pad before returning to its cage. Treatment with neomycin or with control (PBS) started on day 1 and was given intraperitoneally daily for 20 days followed by injection 4 times per week for 42 days. A dosage of 60 mg neomycin per kg body weight was used in this experiment. Mice were examined daily for tumor growth by gentle palpation of the lateral left thorax in the general area of the injection.

6.7.2. RESULTS

As shown in Fig. 4, intraperitoneal treatment with neomycin at 60 mg/kg body weight completely inhibited the establishment of MDA-MB-435 human breast tumors in athymic mice. By day 56, all the mice in the control group (8 mice) receiving only PBS developed tumors, whereas, in the neomycin-treated group, none of the mice had tumors. There was no sign of toxic side effects at this neomycin dosage (60 mg/kg body weight) when administered intraperitoneally for 62 days.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

1. A method for treating a subject having an angiogenesis-related disease, which method comprises administering to the subject a therapeutic amount of (a) neomycin or an analogue thereof sufficient to inhibit the pathological angiogenesis or proliferation of endothelial cells that is associated with the angiogenesis-related disease.

2. The method according to claim 1, wherein the neomycin analogue is

(a) neomycin A, neomycin B, or neomycin C;

(b) a complex comprising neomycin A, neomycin B, or neomycin C;

(c) an aminoglycoside having a structure substantially similar to that of neomycin A, neomycin B or neomycin C;

(d) a chemical or biological breakdown product of neomycin A, neomycin B or neomycin C;

(e) a derivative of neomycin A, neomycin B or neomycin C; or

(f) a naturally-occurring precursor to neomycin A, neomycin B or neomycin C.

3. The method according to claim 2, wherein the neomycin analogue comprises a substituted-2-deoxystreptamine (2-DOS) linked to two to four sugars. wherein each sugar is a pentose or hexose.

4. The method according to claim 3, wherein the neomycin analogue is a member of the neomycin, paromomycin or lividomycin aminoglycoside family.

5. The method according to claim 4, wherein the neomycin analogue comprises a glucosyl residue attached to the 4 position of the 2-DOS moiety, which glucosyl residue comprises an amino group at each of the 2 and 6 positions.

6. The method according to claim 4, wherein the neomycin analogue comprises a 2-DOS and a 2,6-diamino-2-6-dideoxy-D-glucose attached to the 4 position of 2-DOS

7. The method according to claim 5, wherein the neomycin analogue is nebramine, gentamine C_1, gentamine C_2, gentamine C_{1a}, ribostamycin, or xylostasin.

8. The method according to claim 1, wherein the neomycin analogue is an inhibitor of phospholipase C.

9. The method according to claim 1, wherein the neomycin analogue is an inhibitor of nuclear translocation of an angiogenic factor.

10. The method according to claim 1, wherein the neomycin analogue is an inhibitor of endothelial cell proliferation induced by an angiogenic factor.

11. The method according to claim 1, wherein the neomycin analogue is an inhibitor of angiogenesis in the chorioallantoic membrane of chick embryo induced by an angiogenic factor.

12. The method according to claim 9, 10, or 11, wherein the angiogenic factor is an acidic fibroblast growth factor, basic fibroblast growth factor, epidermal growth factor, tumor growth factor-alpha, tumor growth factor-beta, tumor necrosis factor-alpha or vascular endothelial growth factor.

13. The method according to claim 1 in which the subject is a human.

14. The method according to claim 1 in which the angiogenesis-related disease is selected from the group consisting of fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma,

endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma, acoustic neuroma, neurofibroma, trachoma and pyogenic granulomas.

15. The method according to claim 14 wherein the angiogenesis-related disease is breast cancer.

16. The method according to claim 14 wherein the angiogenesis-related disease is prostate cancer.

17. The method according to claim 1 in which the angiogenesis-related disease is selected from the group consisting of acute lymphocytic leukemia and acute myelocytic leukemia, chronic leukemia, polycythemia vera, lymphoma, multiple myeloma, Waldenström's macroglobulinemia, and heavy chain disease.

18. The method according to claim 1 in which the angiogenesis-related disease is selected from the group consisting of acne rosacea, atopic keratitis, bacterial ulcers, chemical burns, contact lens overwear, corneal graft rejection, diabetic retinopathy, epidemic keratoconjunctivitis, fungal ulcers, Herpes simplex infections, herpes zoster infections, Kaposi sarcoma, lipid degeneration, marginal keratolysis, mycobacteria infections, Mooren ulcer, neovascular glaucoma and retrolental

fibroplasia, periphigoid radial keratotomy, phylectenulosis, polyarteritis, protozoan infections, pterygium keratitis sicca, retinopathy of prematurity, rheumatoid arthritis, sjogrens, scleritis, Steven's Johnson disease, superior limbic keratitis, syphilis, systemic lupus, Terrien's marginal degeneration, trauma, vitamin A deficiency, and Wegeners sarcoidosis.

19. The method according to claim 1 in which the angiogenesis-related disease is selected from the group consisting of artery occlusion, Bechets disease, Bests disease, chronic retinal detachment, chronic uveitis/vitritis, carotid obstructive disease, diabetic retinopathy, Eales disease, hyperviscosity syndromes, retinitis, choroiditis, Lyme's disease, macular degeneration, mycobacterial infections, optic pits, Pagets disease, pars planitis, post-laser complications, presumed ocular histoplasmosis, pseudoxanthoma elasticum, retinopathy of prematurity, sickle cell anemia, sarcoid, Stargarts disease, syphilis, systemic lupus erythematosis, toxoplasmosis, trauma, vein occlusion, rubeosis, and proliferative vitreoretinopathy.

20. The method according to claim 1 in which the angiogenesis-related disease is selected from the group consisting of Crohn's disease and ulcerative colitis, psoriasis, rheumatoid arthritis, sarcoidosis, hemangiomas, Osler-Weber-Rendu disease, hereditary hemorrhagic telangiectasia, and acquired immune deficiency syndrome.

21. The method according to claim 14, 15, 16, or 17 which comprises additionally administering an anti-neoplastic agent.

22. The method according to claim 21, wherein the anti-neoplastic agent is selected from the group consisting of docetaxel, etoposide, trontecan, paclitaxel, teniposide, topotecan, vinblastine, vincristine, and vindesine, busulfan, improsulfan, piposulfan, aziridines, benzodepa, carboquone, meturedepa, uredepa, altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide,

chlorambucil, chloraphazine, cyclophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, perfosfamide, phenesterine, prednimustine, trofosfamide, uracil mustard, carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine, dacarbazine, mannomustine, mitobronitol, mitolactol, pipobroman, temozolomide, aclacinomycinsa actinomycin F_1, anthramycin, azaserine, bleomycins, cactinomycin, carubicin, carzinophilin, chromomycins, dactinomycin, daunorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, idarubicin, menogaril, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, pirarubicin, plicamycin, porfiromycin, puromycin, streptonigrin, streptozocin, tubercidin, zinostatin, zorubicin, denopterin, edatrexate, methotrexate, piritrexim, pteropterin, Tomudex®, trimetrexate, cladribine, fludarabine, 6-mercaptopurine, thiamiprine, thioguanine, ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, doxifluridine, emitefur, enocitabune, floxuridine, fluorouracil, gemcitabine, tegafur, L-asparaginase, interferon-α, interferon-β, interferon-γ, interleukin-2, lentinan, propagermanium, PSK, roquinimex, sizofican, ubenimex, carboplatin, cisplatin, miboplatin, oxaliplatin, aceglarone, amsacrine, bisantrene, defosfamide, demecolcine, diaziquone, eflornithine, elliptinium acetate, etoglucid, fenretinide, gallium nitrate, hydroxyurea, lonidamine, miltefosine, mitoguazone, mitoxantrone, mopidamol, nitracine, pentostain, phenamet, podophyllinic acid 2-ethyl-hydrazide, procabazine, razoxane, sobuzoxane, spirogermanium, tenuzonic acid, triaziquone, 2,2',2"trichlorotriethylamine, urethan, calusterone, dromostanolone, epitiostanol, mepitiostane, testolacone, aminoglutethimide, mitotane, trilostane, bicalutamide, flutamide, nilutamide, droloxifene, tamoxifen, toremifene, aminoglutethimide, anastrozole, fadrozole, formestane, letrozole, fosfestrol, hexestrol, polyestradiol phosphate, buserelin, goserelin, leuprolide, triptorelin, chlormadinone acetate, medroxyprogesterone, megestrol acetate, melengestrol, porfimer sodium, batimastar, and folinic acid.

23. A method for treating a subject having an angiogenesis-related disease, which method comprises administering to the subject a therapeutic amount of (a)

neomycin or an analogue thereof, and (b) an anti-angiogenic agent that is not neomycin or an analogue thereof, sufficient to inhibit the pathological angiogenesis or proliferation of endothelial cells that is associated with the angiogenesis-related disease.

24. The method according to claim 23, wherein the anti-angiogenic agent is selected from the group consisting of thalidomide, 2-methoxyestradiol, endostatin, angiostatin, platelet factor-4, dextran sulfate, beta-1,3-glucan sulfate, interferon-alpha, interleukin-12, 22-oxa-1 α, 25-dihydroxyvitamin D_2, monoclonal antibody 26-2F, monoclonal antibody 36U, peptide comprising the sequence NH_2-Val-Phe-Ser-Val-Arg-Val-Ser-Ile-Leu-Val-Phe-COOH, peptide comprising the sequence NH_2-Leu-Leu-Phe-Leu-Pro-Leu-Gly-Val-Ser-Leu-Leu-Asp-Ser-COOH, human placental ribonuclease inhibitor, peptide comprising the sequence NH_2-Tyr-Ser-Val-Trp-Ile-Gly-Gly-Ser-Ile-Leu-Ala-Ser-Leu-Ser-Thr-Phe-Gln-Gln-Met-Trp-Ile-Ser-Lys-COOH, peptide comprising the sequence NH_2-Ala-Gln-Leu-Ala-Gly-Glu-Cys-Arg-Glu-Asn-Val-Cys-Met-Gly-Ile-Glu-Gly-Arg-COOH, nucleotide comprising the sequence 5'-CGGACGAATGCTTTGATGTTGTGCTGGACCAGCGTTCATTCTCA-3', anthracycline, 15-deoxyspergualin, D-penicillamine, eponemycin, fumagillin, AGM-1470, herbimycin A, rapamycin, CAI, CM101, and marimastat.

25. A pharmaceutical composition for treating a subject having an angiogenesis-related disease comprising an therapeutic effective amount of (a) neomycin or an analogue thereof, and (b) an anti-angiogenic agent that is not neomycin or an analogue thereof, in pharmaceutically acceptable form sufficient to suppress the pathological angiogenesis or proliferation of endothelial cells that is associated with the angiogenesis-related disease.

26. The pharmaceutical composition of claim 25, wherein the neomycin analogue is

(a) neomycin A, neomycin B, or neomycin C;

(b) a complex comprising neomycin A, neomycin B, or neomycin C;

(c) an aminoglycoside having a structure substantially similar to that of neomycin A, neomycin B or neomycin C;

(d) a chemical or biological breakdown product of neomycin A, neomycin B or neomycin C;

(e) a derivative of neomycin A, neomycin B or neomycin C; or

(f) a naturally-occurring precursor to neomycin A, neomycin B or neomycin C.

27. The pharmaceutical composition of claim 26, wherein the neomycin analogue comprises a substituted-2-deoxystreptamine (2-DOS) linked to two to four sugars, wherein each sugar is a pentose or hexose.

28. The pharmaceutical composition of claim 27, wherein the neomycin analogue is a member of the neomycin, paromomycin or lividomycin aminoglycoside family.

29. The pharmaceutical composition of claim 28, wherein the neomycin analogue comprises a glucosyl residue attached to the 4 position of the 2-DOS moiety, which glucosyl residue comprises an amino group at each of the 2 and 6 positions.

30. The pharmaceutical composition of claim 29, wherein the neomycin analogue comprises a 2-DOS and a 2,6-diamino-2-6-dideoxy-D-glucose attached to the 4 position of 2-DOS.

31. The pharmaceutical composition of claim 29, wherein the neomycin analogue is nebramine, gentamine C_1, gentamine C_2, gentamine C_{1a}, ribostamycin, or xylostasin.

32. The pharmaceutical composition of claim 25, wherein the neomycin analogue is an inhibitor of nuclear translocation of an angiogenic factor.

33. The pharmaceutical composition of claim 25, wherein the neomycin analogue is an inhibitor of phospholipase C.

34. The pharmaceutical composition of claim 25, wherein the neomycin analogue is an inhibitor of endothelial cell proliferation induced by an angiogenic factor.

35. The pharmaceutical composition of claim 25, wherein the neomycin analogue is an inhibitor of angiogenesis in the chorioallantoic membrane of chick embryo induced by an angiogenic factor.

36. The pharmaceutical composition of claim 32, 34 or 35, wherein the angiogenic factor is an acidic fibroblast growth factor, basic fibroblast growth factor, epidermal growth factor, tumor growth factor-alpha, tumor growth factor-beta, tumor necrosis factor-alpha, vascular endothelial growth factor, platelet-derived growth factor, platelet-derived endothelial cell growth fqactor, placental growth factor, hepatocyte growth fctor, platelet activating factor, insulin-like growth factor, interleukin-8, or granulocyte-colony stimulating factor.

37. The pharmaceutical composition of claim 25, further comprising a pharmaceutically acceptable carrier.

38. The pharmaceutical composition of claim 25 in which the subject is a human.

39. The pharmaceutical composition of claim 25 in which the angiogenesis-related disease is selected from the group consisting of fibrosarcoma, myxosarcoma,

liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma, acoustic neuroma, neurofibroma, trachoma and pyogenic granulomas.

40. The pharmaceutical composition of claim 25 in which the angiogenesis-related disease is selected from the group consisting of acute lymphocytic leukemia and acute myelocytic leukemia, chronic leukemia, polycythemia vera, lymphoma, multiple myeloma, Waldenström's macroglobulinemia, and heavy chain disease.

41. The pharmaceutical composition of claim 25 in which the angiogenesis-related disease is selected from the group consisting of acne rosacea, atopic keratitis, bacterial ulcers, chemical burns, contact lens overwear, corneal graft rejection, diabetic retinopathy, epidemic keratoconjunctivitis, fungal ulcers, Herpes simplex infections, herpes zoster infections, Kaposi sarcoma, lipid degeneration, marginal keratolysis, mycobacteria infections, Mooren ulcer, neovascular glaucoma and retrolental fibroplasia, periphigoid radial keratotomy, phylectenulosis, polyarteritis, protozoan infections, pterygium keratitis sicca, retinopathy of prematurity, rheumatoid arthritis, sjogrens, scleritis, Steven's Johnson disease, superior limbic keratitis, syphilis, systemic lupus, Terrien's marginal degeneration, trauma, vitamin A deficiency, and Wegeners sarcoidosis.

42. The pharmaceutical composition of claim 25 in which the angiogenesis-related disease is selected from the group consisting of artery occlusion, Bechets disease, Bests disease, chronic retinal detachment, chronic uveitis/vitritis, carotid obstructive disease, diabetic retinopathy, Eales disease, hyperviscosity syndromes, retinitis, choroiditis, Lyme's disease, macular degeneration, mycobacterial infections, optic pits, Pagets disease, pars planitis, post-laser complications, presumed ocular histoplasmosis, pseudoxanthoma elasticum, retinopathy of prematurity, sickle cell anemia, sarcoid, Stargarts disease, syphilis, systemic lupus erythematosis, toxoplasmosis, trauma, vein occlusion, rubeosis, and proliferative vitreoretinopathy.

43. The pharmaceutical composition of claim 25 in which the angiogenesis-related disease is selected from the group consisting of Crohn's disease and ulcerative colitis, psoriasis, rheumatoid arthritis, sarcoidosis, hemangiomas, Osler-Weber-Rendu disease, hereditary hemorrhagic telangiectasia, and acquired immune deficiency syndrome.

44. The pharmaceutical composition of claim 25 in which the anti-angiogenic factor is selected from the group consisting of thalidomide, 2-methoxyestradiol, endostatin, angiostatin, platelet factor-4, dextran sulfate, beta-1,3-glucan sulfate, interferon-alpha, interleukin-12, 22-oxa-1 α, 25-dihydroxyvitamin D_2, monoclonal antibody 26-2F, monoclonal antibody 36U, peptide comprising the sequence NH_2-Val-Phe-Ser-Val-Arg-Val-Ser-Ile-Leu-Val-Phe-COOH, peptide comprising the sequence NH_2-Leu-Leu-Phe-Leu-Pro-Leu-Gly-Val-Ser-Leu-Leu-Asp-Ser-COOH, human placental ribonuclease inhibitor, peptide comprising the sequence NH_2-Tyr-Ser-Val-Trp-Ile-Gly-Gly-Ser-Ile-Leu-Ala-Ser-Leu-Ser-Thr-Phe-Gln-Gln-Met-Trp-Ile-Ser-Lys-COOH, peptide comprising the sequence NH_2-Ala-Gln-Leu-Ala-Gly-Glu-Cys-Arg-Glu-Asn-Val-Cys-Met-Gly-Ile-Glu-Gly-Arg-COOH, nucleotide comprising the sequence 5'-
CGGACGAATGCTTTGATGTTGTGCTGGACCAGCGTTCATTCTCA-3',

anthracycline, 15-deoxyspergualin, D-penicillamine, eponemycin, fumagillin, AGM-1470, herbimycin A, rapamycin, CAI, CM101, and marimastat.

45. A pharmaceutical composition comprising a therapeutically effective amount of (a) neomycin or an analogue thereof, and (b) an anti-neoplastic agent, in pharmaceutically acceptable form and in an amount sufficient to treat an angiogenesis-related disease which is a tumor in a subject.

46. The pharmaceutical composition of claim 45, wherein the neomycin analogue is

(a) neomycin A, neomycin B, or neomycin C;

(b) a complex comprising neomycin A, neomycin B, or neomycin C;

(c) an aminoglycoside having a structure substantially similar to that of neomycin A, neomycin B or neomycin C;

(d) a chemical or biological breakdown product of neomycin A, neomycin B or neomycin C;

(e) a derivative of neomycin A, neomycin B or neomycin C; or

(f) a naturally-occurring precursor to neomycin A, neomycin B or neomycin C.

47. The pharmaceutical composition of claim 46, wherein the neomycin analogue comprises a substituted-2-deoxystreptamine (2-DOS) linked to two to four sugars, wherein each sugar is a pentose or hexose.

48. The pharmaceutical composition of claim 47, wherein the neomycin analogue is a member of the neomycin, paromomycin or lividomycin aminoglycoside family.

49. The pharmaceutical composition of claim 48, wherein the neomycin analogue comprises a glucosyl residue attached to the 4 position of the 2-DOS moiety, which glucosyl residue comprises an amino group at each of the 2 and 6 positions.

50. The pharmaceutical composition of claim 49, wherein the neomycin analogue comprises a 2-DOS and a 2,6-diamino-2-6-dideoxy-D-glucose attached to the 4 position of 2-DOS.

51. The pharmaceutical composition of claim 49, wherein the neomycin analogue is nebramine, gentamine C_1, gentamine C_2, gentamine C_{1a}, ribostamycin, or xylostasin.

52. The pharmaceutical composition of claim 45, wherein the neomycin analogue is an inhibitor of nuclear translocation of an angiogenic factor.

53. The pharmaceutical composition of claim 45, wherein the neomycin analogue is an inhibitor of phospholipase C.

54. The pharmaceutical composition of claim 45, wherein the neomycin analogue is an inhibitor of endothelial cell proliferation induced by an angiogenic factor.

55. The pharmaceutical composition of claim 45, wherein the neomycin analogue is an inhibitor of angiogenesis in the chorioallantoic membrane of chick embryo induced by an angiogenic factor.

56. The pharmaceutical composition of claim 52, 54 or 55, wherein the angiogenic factor is an acidic fibroblast growth factor, basic fibroblast growth factor, epidermal growth factor, tumor growth factor-alpha, tumor growth factor-beta, tumor necrosis factor-alpha, vascular endothelial growth factor, platelet-derived growth

factor, platelet-derived endothelial cell growth fqactor, placental growth factor, hepatocyte growth fctor, platelet activating factor, insulin-like growth factor, interleukin-8, or granulocyte-colony stimulating factor.

57. The pharmaceutical composition of claim 45, further comprising a pharmaceutically acceptable carrier.

58. The pharmaceutical composition of claim 45 in which the subject is a human.

59. The pharmaceutical composition of claim 45 in which the angiogenesis-related disease is selected from the group consisting of fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma, acoustic neuroma, neurofibroma, trachoma and pyogenic granulomas.

60. The pharmaceutical composition of claim 59 wherein the angiogenesis-related disease is breast cancer.

61. The pharmaceutical composition of claim 59 wherein the angiogenesis-related disease is prostate cancer.

62. The pharmaceutical composition of claim 45 in which the angiogenesis-related disease is selected from the group consisting of acute lymphocytic leukemia and acute myelocytic leukemia, chronic leukemia, polycythemia vera, lymphoma, multiple myeloma, Waldenström's macroglobulinemia, and heavy chain disease.

63. The pharmaceutic composition of claim 45, wherein the anti-neoplastic agent is selected from the group consisting of docetaxel, etoposide, trontecan, paclitaxel, teniposide, topotecan, vinblastine, vincristine, and vindesine, busulfan, improsulfan, piposulfan, aziridines, benzodepa, carboquone, meturedepa, uredepa, altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide, chlorambucil, chloraphazine, cyclophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, perfosfamide, phenesterine, prednimustine, trofosfamide, uracil mustard, carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine, dacarbazine, mannomustine, mitobronitol, mitolactol, pipobroman, temozolomide, aclacinomycinsa actinomycin F_1, anthramycin, azaserine, bleomycins, cactinomycin, carubicin, carzinophilin, chromomycins, dactinomycin, daunorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, idarubicin, menogaril, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, pirarubicin, plicamycin, porfiromycin, puromycin, streptonigrin, streptozocin, tubercidin, zinostatin, zorubicin, denopterin, edatrexate, methotrexate, piritrexim, pteropterin, Tomudex®, trimetrexate, cladribine, fludarabine, 6-mercaptopurine, thiamiprine, thioguanine, ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, doxifluridine, emitefur, enocitabune, floxuridine, fluorouracil, gemcitabine, tegafur, L-asparaginase, interferon-α, interferon-β, interferon-γ, interleukin-2, lentinan, propagermanium, PSK, roquinimex, sizofican, ubenimex, carboplatin, cisplatin, miboplatin, oxaliplatin, aceglarone, amsacrine, bisantrene, defosfamide, demecolcine, diaziquone,

eflornithine, elliptinium acetate, etoglucid, fenretinide, gallium nitrate, hydroxyurea, lonidamine, miltefosine, mitoguazone, mitoxantrone, mopidamol, nitracine, pentostain, phenamet, podophyllinic acid 2-ethyl-hydrazide, procabazine, razoxane, sobuzoxane, spirogermanium, tenuzonic acid, triaziquone, 2,2',2"trichlorotriethylamine, urethan, calusterone, dromostanolone, epitiostanol, mepitiostane, testolacone, aminoglutethimide, mitotane, trilostane, bicalutamide, flutamide, nilutamide, droloxifene, tamoxifen, toremifene, aminoglutethimide, anastrozole, fadrozole, formestane, letrozole, fosfestrol, hexestrol, polyestradiol phosphate, buserelin, goserelin, leuprolide, triptorelin, chlormadinone acetate, medroxyprogesterone, megestrol acetate, melengestrol, porfimer sodium, batimastar, and folinic acid.

64. A method for selecting a neomycin analogue for use in treating an angiogenesis-related disease, comprising testing the neomycin analogue for activity for inhibiting angiogenesis.

65. The method according to claim 64, which comprises

(a) incubating a first culture of endothelial cells with the neomycin analogue and an angiogenic factor in a growth medium, and incubating a second culture of endothelial cells with the angiogenic factor in the growth medium lacking the neomycin analogue, wherein the angiogenic factor is labeled;

(b) determining the amounts of angiogenic factor present in the nuclei of cells in the first and the second cultures; and

(c) selecting for use in treating the angiogenesis-related disease, the neomycin analogue that inhibits nuclear translocation of the angiogenic factor in cells of the first culture by at least 10% of the amount of the angiogenic factor translocated to the nuclei of the cells in the second culture.

66. The method according to claim 64, which comprises

(a) incubating a first culture of endothelial cells with the neomycin analogue in a growth medium, and incubating a second culture of endothelial cells in a growth medium lacking the neomycin analogue;

(b) incubating the first and the second cultures with an angiogenic factor in the growth medium, wherein the angiogenic factor is labeled;

(c) determining the amount of angiogenic factor present in the nuclei of cells in the first and the second cultures; and

(d) selecting for use in treating the angiogenesis-related disease, the neomycin analogue that inhibits nuclear translocation of the angiogenic factor in the cells of the first culture by at least 10% of the amount of nuclear translocation of the angiogenic factor in the cells of the second culture.

67. The method according to claim 64, which comprises

(a) incubating a first culture of endothelial cells with the neomycin analogue and an angiogenic factor in a growth medium, incubating a second culture of endothelial cells with the neomycin analogue in the growth medium lacking the angiogenic factor, incubating a third culture of endothelial cells with the angiogenic factor in the growth medium lacking the neomycin analogue, incubating a fourth culture of endothelial cells in the growth medium lacking the neomycin analogue and the angiogenic factor;

(b) determining the cell numbers of the first, the second, the third and the fourth cultures; and

(c) selecting for use in treating the angiogenesis-related disease, the neomycin analogue that reduces the increase in the cell number in the second culture over the cell number in the first culture to less than about 75% of the increase in cell number of the third culture over the cell number of the fourth culture.

68. The method according to claim 64, which comprises

(a) contacting the chorioallantoic membrane of a first group of chick embryos with the neomycin analogue and an angiogenic factor, contacting the chorioallantoic membrane of a second group of chick embryos with the neomycin analogue but not the angiogenic factor, contacting the chorioallantoic membrane of a third group of chick embryos with the angiogenic factor but not the neomycin analogue, and contacting the chorioallantoic membrane of a fourth group of chick embryos with a solution lacking the neomycin analogue and the antigenic factor;

(b) incubating the first, the second, the third and the fourth groups of chick embryos;

(c) determining the numbers of embryos having an angiogenic response in the first, the second, the third and the fourth groups of embryos; and

(d) selecting for use in treating the angiogenesis-related disease, the neomycin analogue that reduces the increase in the number of embryos exhibiting an angiogenic response in the second group of embryos over the number of embryos exhibiting an angiogenic response in the first group of embryos to less than about 75% of the increase in the number of embryos exhibiting an angiogenic response in the third group of embryos over the number of embryos exhibiting an angiogenic response in the fourth group of embryos.

69. The method according to any one of claims 64 to 68, wherein the neomycin analogue is

(a) neomycin A, neomycin B, or neomycin C;

(b) a complex comprising neomycin A, neomycin B, or neomycin C;

(c) an aminoglycoside having a structure substantially similar to that of neomycin A, neomycin B or neomycin C;

(d) a chemical or biological breakdown product of neomycin A, neomycin B or neomycin C;

(e) a derivative of neomycin A, neomycin B or neomycin C; or

(f) a naturally-occurring precursor to neomycin A, neomycin B or neomycin C.

70. The method according to claim 69, wherein the neomycin analogue comprises a substituted-2-deoxystreptamine (2-DOS) linked to two to four sugars, wherein each sugar is a pentose or hexose.

71. The method according to claim 70, wherein the neomycin analogue is a member of the neomycin, paromomycin or lividomycin aminoglycoside family.

72. The method according to claim 71, wherein the neomycin analogue comprises a glucosyl residue attached to the 4 position of the 2-DOS moiety, which glucosyl residue comprises an amino group at each of the 2 and 6 positions.

73. The method according to claim 72, wherein the neomycin analogue comprises a 2-DOS and a 2,6-diamino-2-6-dideoxy-D-glucose attached to the 4 position of 2-DOS

74. The method according to any one of claims 64 to 68, wherein the angiogenic factor is an acidic fibroblast growth factor, basic fibroblast growth factor, epidermal growth factor, tumor growth factor-alpha, tumor growth factor-beta, tumor necrosis factor-alpha, vascular endothelial growth factor, platelet-derived growth factor, platelet-derived endothelial cell growth fqactor, placental growth factor, hepatocyte growth fctor, platelet activating factor, insulin-like growth factor, interleukin-8, or granulocyte-colony stimulating factor.

75. The method according to any one of claims 64 to 68, in which the angiogenesis-related disease is selected from the group consisting of fibrosarcoma,

myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma, acoustic neuroma, neurofibroma, trachoma and pyogenic granulomas.

76. The method according to any one of claims 64 to 68 wherein the angiogenesis-related disease is breast cancer.

77. The method according to any one of claims 64 to 68 wherein the angiogenesis-related disease is prostate cancer.

78. The method according to any one of claims 64 to 68, in which the angiogenesis-related disease is selected from the group consisting of acute lymphocytic leukemia and acute myelocytic leukemia, chronic leukemia, polycythemia vera, lymphoma, multiple myeloma, Waldenström's macroglobulinemia, and heavy chain disease.

79. The method according to any one of claims 64 to 68, in which the angiogenesis-related disease is selected from the group consisting of acne rosacea, atopic keratitis, bacterial ulcers, chemical burns, contact lens overwear, corneal graft

rejection, diabetic retinopathy, epidemic keratoconjunctivitis, fungal ulcers, Herpes simplex infections, Herpes zoster infections, Kaposi sarcoma, lipid degeneration, marginal keratolysis, mycobacteria infections, Mooren ulcer, neovascular glaucoma and retrolental fibroplasia, periphigoid radial keratotomy, phylectenulosis, polyarteritis, protozoan infections, pterygium keratitis sicca, retinopathy of prematurity, rheumatoid arthritis, sjogrens, scleritis, Steven's Johnson disease, superior limbic keratitis, syphilis, systemic lupus, Terrien's marginal degeneration, trauma, Vitamin A deficiency, and Wegeners sarcoidosis.

80. The method according to any one of claims 64 to 68, in which the angiogenesis-related disease is selected from the group consisting of artery occlusion, Bechets disease, Bests disease, chronic retinal detachment, chronic uveitis/vitritis, carotid obstructive disease, diabetic retinopathy, Eales disease, hyperviscosity syndromes, retinitis, choroiditis, Lyme's disease, macular degeneration, mycobacterial infections, optic pits, Pagets disease, pars planitis, post-laser complications, presumed ocular histoplasmosis, pseudoxanthoma elasticum, retinopathy of prematurity, sickle cell anemia, sarcoid, Stargarts disease, syphilis, systemic lupus erythematosis, toxoplasmosis, trauma, vein occlusion, rubeosis, and proliferative vitreoretinopathy.

81. The method according to any one of claims 64 to 68, in which the angiogenesis-related disease is selected from the group consisting of Crohn's disease and ulcerative colitis, psoriasis, rheumatoid arthritis, sarcoidosis, hemangiomas, Osler-Weber-Rendu disease, hereditary hemorrhagic telangiectasia, and acquired immune deficiency syndrome.

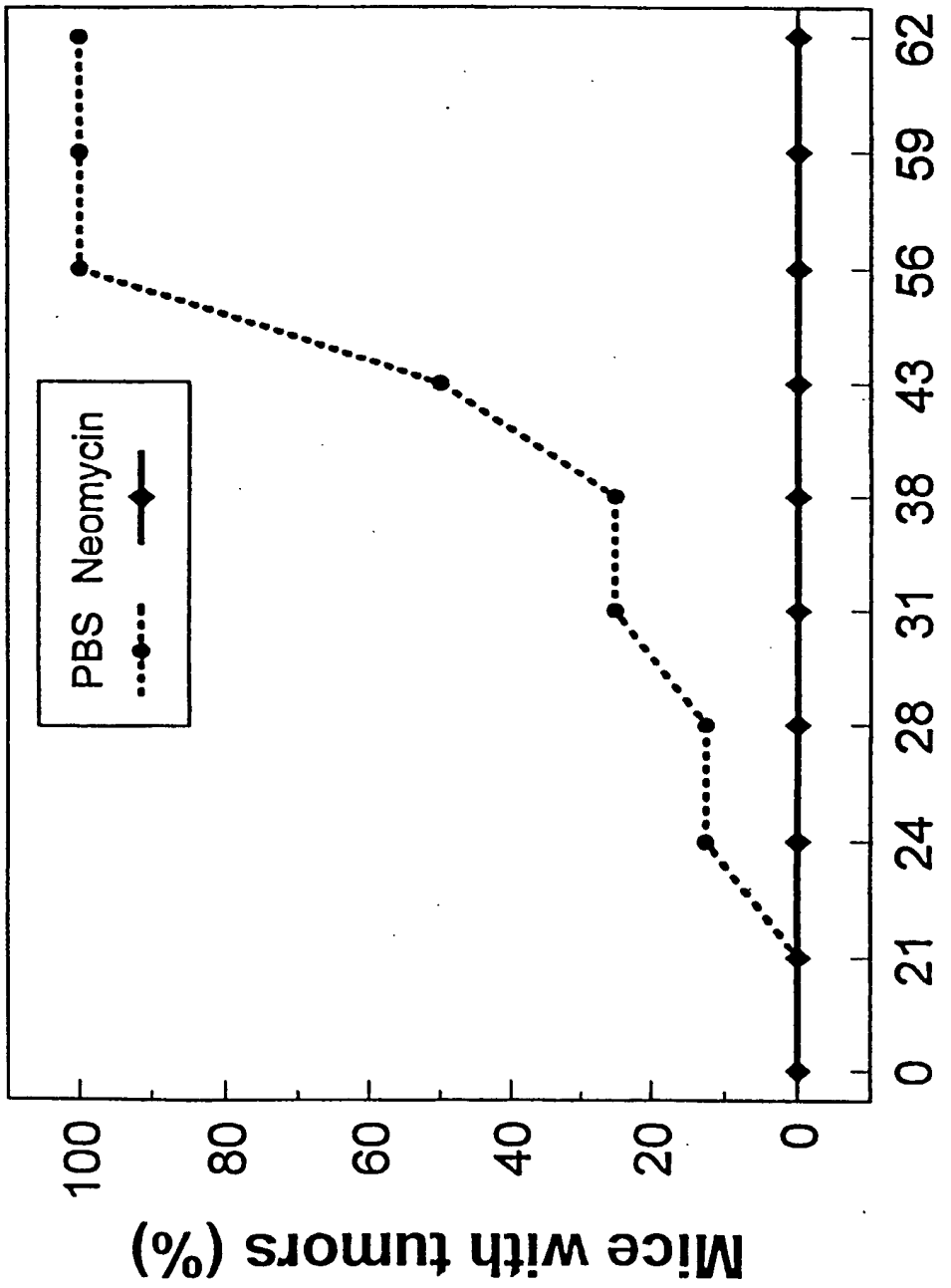
Mice with tumors (%)
100
80
60
40
20
0
0
21
24
28
31
38
43
56
59
62
Days
PBS
Neomycin

FIG.1

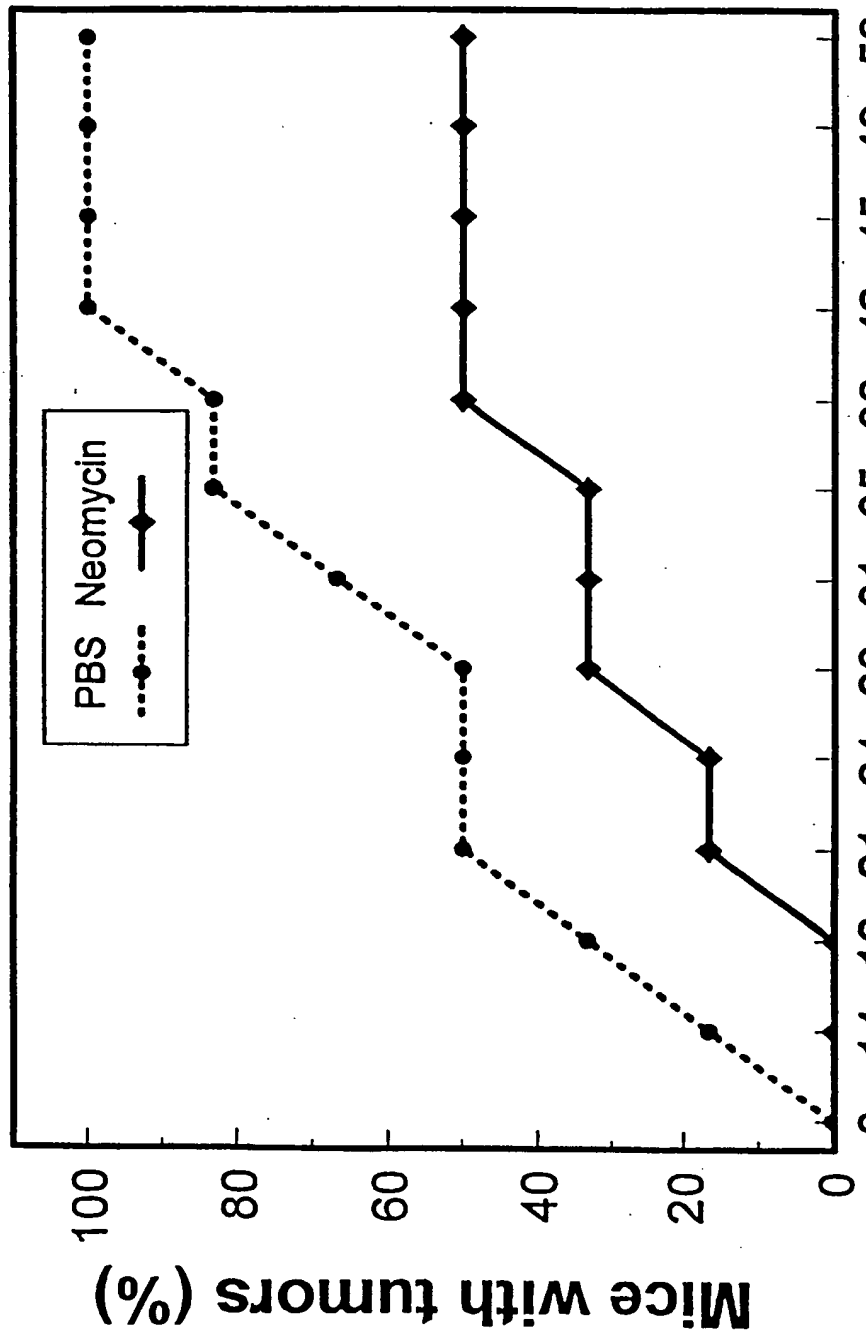
Mice with tumors (%)
100
80
60
40
20
0
0 14 18 21 24 28 31 35 38 42 45 49 59
Days
PBS
Neomycin

FIG.2

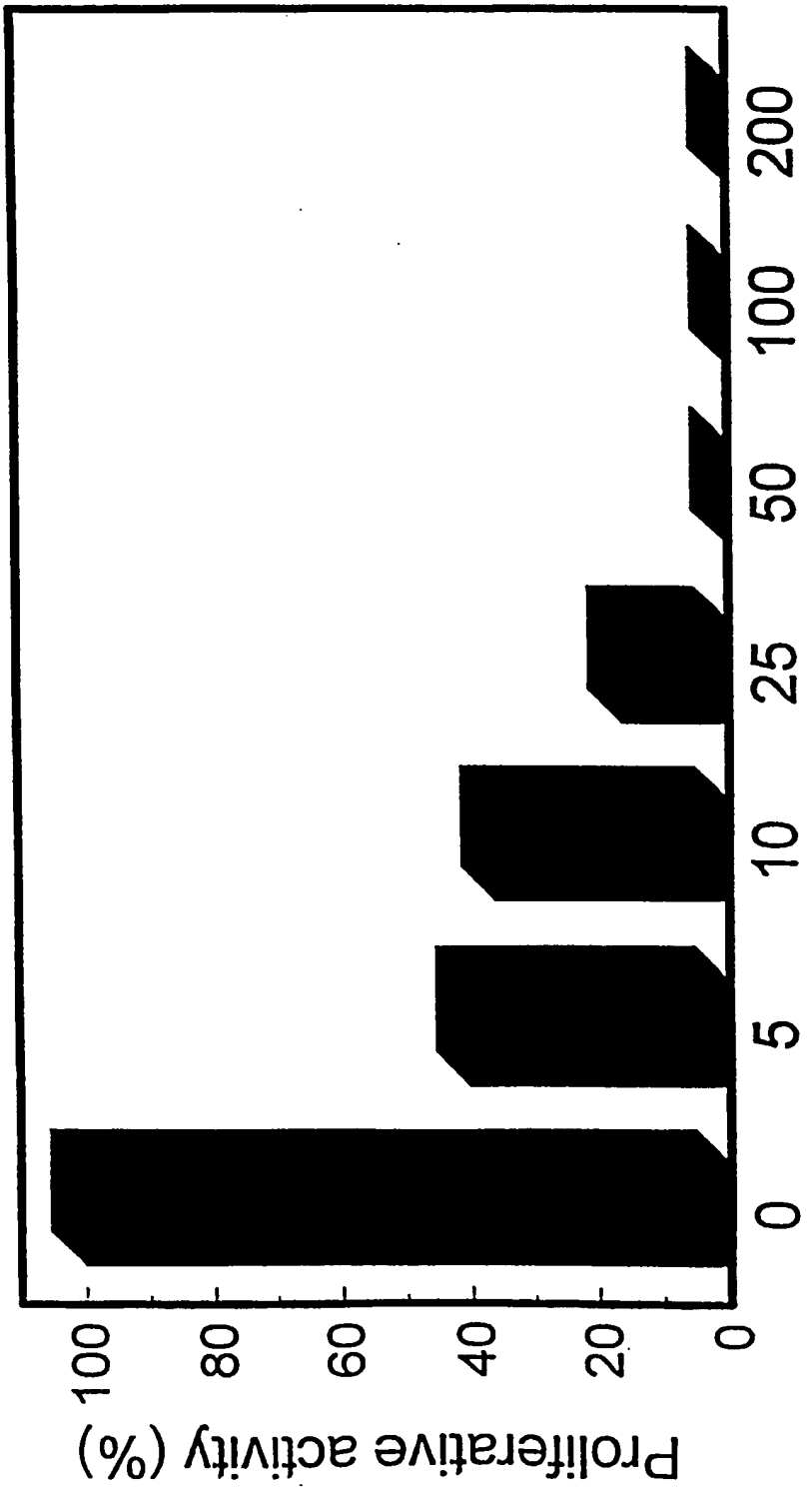
Proliferative activity (%)
100
80
60
40
20
0
0
5
10
25
50
100
200
Neomycin (μM)

FIG.3

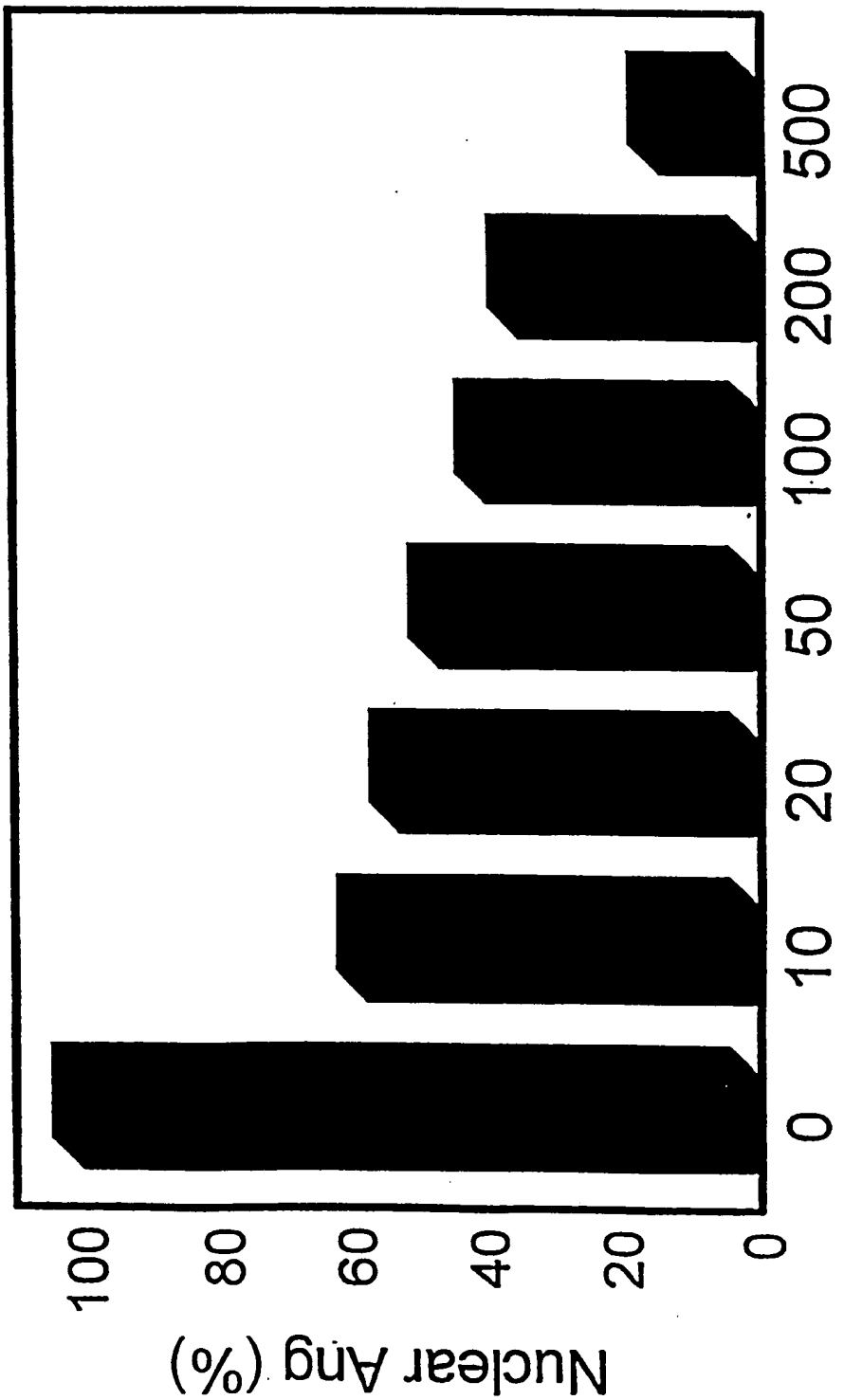

FIG.4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/10269

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/37
US CL :514/39; 536/13.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/39; 536/13.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2,799,620 A (WAKSMAN et al.) 16 July 1957, whole document.	25-44, 45-63

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 13 SEPTEMBER 1999	Date of mailing of the international search report 26 OCT 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer RICHARD L. RAYMOND Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet)(July 1992)*